CHARACTERIZATION OF THE ANTI-TUMOR EFFECTS OF IFNγ AND SUPERANTIGENS IN THE TREATMENT OF CANCER

By

SCOTT LEE KOMINSKY

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2000

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Howard M. Johnson for giving me the opportunity to study in his laboratory. He has been instrumental in my development as a scientist. Dr. Johnson has given me expert guidance in my studies as I worked toward the completion of my degree and has provided invaluable advice to help me forge a rewarding career path. I would also like to thank my committee members, Dr. Edward Hoffmann, Dr. Francis Davis, Dr. Janet Yamamoto, and Dr. Ammon Peck, for their advice, time, and patience. A special note of thanks goes to Dr. Barbara Torres who has played an integral part in helping me with my studies and has greatly contributed to my scientific education. I would also like to thank Dr. Prem Subramaniam for his help in general over the years as well as for his expert assistance in cell cycle studies. I would like to acknowledge Melissa Chen and Dr. Neal Benson for their expert assistance in flow cytometry studies. I would especially like to acknowledge Dr. Amy Hobeika who played a major role in helping me with just about everything. Many special thanks go to my labmates: Martez, Taishi, Karrie, Joe, George, and Mustafa. They have been helpful in my studies and have been good friends. A special note of thanks also goes out to Gail Bryan and Faith Lake who have both provided excellent assistance in my studies.

I would especially like to thank my family for their support and guidance. My father, uncle, and grandmother were always willing to come to Gainesville to

visit me when I did not have time to travel to visit them. They also played an integral part in keeping my refrigerator full as did my mother. I would also like to thank my sister Jamee for making me laugh and cleaning my apartment on occasion. They have always believed in me and made being in school much easier. A special note of thanks goes to my friends Chris, Mike, Anna, and Joshua for their ability to keep my spirits up and help me to relax and have a good time. Lastly, I would especially like to thank my fiancée Kelly. She has helped me through the whole journey providing tremendous encouragement and support.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ii

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT xi

CHAPTERS

1 INTRODUCTION 1

Cancer and Cancer Treatment 1
Discovery and Biological Activities of IFNγ 4
IFNγ and Disease 9
IFNγ and Signal Transduction 10
The Mammalian Cell Cycle and Cancer 12
Nucleoside Analogs and Disease 24
Growth Factor Receptors and Transcriptional Co-activators 25
Discovery and Biological Activity of Superantigens 27

2 MATERIALS AND METHODS 30

Reagents, Cell Lines, and Plasmids 30
Antiproliferation Assay 31
Cell Cycle Analysis 32
CTL Assay 32
ELISA for IFNγ 33
Expansion of $CD4^+$, $CD8^+$, and NK Cells 33
Immunoprecipitation and Immunoblotting 34
In Vitro Kinase Assays 34
In Vivo Vaccination: Tumor Challenge Experiments 35
Transfection 36

3 RESULTS 37

IFNγ Inhibition of the Glioblastoma Cell Cycle 37
Inhibition of Cell Growth 37
Cell Cycle Analysis 40
Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$ .. 44
Induction of $p21^{WAF1/CIP1}$ Leads to Increased Binding of $p21^{WAF1/CIP1}$ to Cdk2 47
Inhibition of Cdk2 Activity 50
Combined Inhibitory Effect of IFNγ and Acyclovir on the Glioblastoma Cell Cycle 54
IFNγ Inhibition of Cell Growth 54
Acyclovir Inhibition of Cell Growth 56
Phosphorylation of Acyclovir by Cellular Kinases 56
Cell Cycle Analysis Following Acyclovir Treatment 59
Combined Effect of IFNγ and Acyclovir on Cell Growth 61
Cell Cycle Analysis Following IFNγ and Acyclovir Treatment... 71
IFNγ Induction of $p21^{WAF1/CIP1}$ Leads to Increased Binding of $p21^{WAF1/CIP1}$ to PCNA 77
IFNγ Inhibition of Prostate Cancer Cell Proliferation 79
Inhibition of Cell Growth 79
Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$... 82
IFNγ-Induced Phosphorylation of STAT1 82
Downregulation of neu/HER-2 84
Effect of p300 on IFNγ-Induced neu/HER-2 Downregulation..... 88
Effect of p300 on IFNγ Antiproliferative Activity 91
Effect of p300 on IFNγ-Induced $p21^{WAF1/CIP1}$ Production 91
Superantigen Amplification of Anti-Tumor Immunity 94
Vaccination Followed By Superantigen Administration Elicits Anti-Tumor Protection 94
Protection of Long Term Survivors Against Rechallenge With Viable Tumor 96
Effect of Superantigen Dose on Protection Against Tumor Challenge 96
Expansion of $CD4^+$, $CD8^+$, and Natural Killer Cells Following Vaccination and Superantigen Administration 100
Vaccination Followed by Superantigen Administration Leads to Increased CTL Activity 103
Effect of Superantigen Administration on Serum Levels of IFNγ 105
IFNγ Inhibition of B16F10 Cell Growth 108
Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$... 108
Vaccination Followed by Superantigen Administration Induces a Specific Immune Response 111
Protection of Mice With Established Tumor by Superantigen ... 111

4 DISCUSSION 115

Inhibition of the Glioblastoma Cell Cycle by IFNγ and Acyclovir 115
IFNγ Inhibition of Prostate Cancer Cell Proliferation 120
Superantigen Amplification of Anti-Tumor Immunity 123

REFERENCE LIST 129

BIOGRAPHICAL SKETCH 139

LIST OF TABLES

Table		page
1-1	Properties of oncogenes and tumor suppressor genes	2
1-2	Properties of IFNγ	6
1-3	Biological Activities of IFNγ	7
1-4	Cyclin-dependent Kinase Inhibitors	19
3-1	Inhibition of Glioblastoma Cell Line Growth	39
3-2	Effects of IFNγ on Cdk2 Activity in T98G	51
3-3	Effects of IFNγ on Cdk2 Activity in SNB-19	52
3-4	Effects of IFNγ on Cdk2 Activity in U-373	53
3-5	Inhibition of Glioblastoma Cell Line Proliferation	58
3-6	Cell Cycle Analysis of Acyclovir-Treated T98G Cells	62
3-7	Cell Cycle Analysis of Acyclovir-Treated SNB-19 Cells	63
3-8	Cell Cycle Analysis of Acyclovir-Treated U-373 Cells	64
3-9	Cell Cycle Analysis of IFNγ and Acyclovir-Treated T98G Cells	74
3-10	Cell Cycle Analysis of IFNγ and Acyclovir-Treated SNB-19 Cells	75
3-11	Cell Cycle Analysis of IFNγ and Acyclovir-Treated U-373 Cells	76
3-12	Inhibition of Prostate Cancer Cell Growth by IFNγ	80
3-13	Inhibition of PC-3 (pRc/RSV-p300) Cell Growth by IFNγ	92
3-14	Expansion of $CD4^+$, $CD8^+$, and NK Cells Following Vaccination and Superantigen Treatment	104

LIST OF FIGURES

Figure		page
1-1	The IFNγ Signal Transduction Pathway	11
1-2	The Mammalian Cell Cycle	14
1-3	The Role of p53 in Guarding Against Tumor Formation	23
3-1	IFNγ Inhibits Cellular Proliferation of Glioblastoma Cell Lines T98G, SNB-19, and U-373 in a Dose-Dependent Manner	38
3-2	IFNγ Inhibits the Progression of T98G Cells Through the Cell Cycle Causing a Strong G_1 Delay	41
3-3	IFNγ Inhibits the Progression of U-373 Cells Through the Cell Cycle Causing a G_1 Delay	42
3-4	IFNγ Inhibits the Progression of SNB-19 Cells Through the Cell Cycle Causing a Strong G_1-S Delay	43
3-5	IFNγ Treatment of Synchronized Cells (by serum starvation) Increases $p21^{WAF1/CIP1}$ Production in (a) T98G, (b) SNB-19, and (c) U-373 Cells	45
3-6	IFNγ Treatment Increases $p21^{WAF1/CIP1}$ Production	46
3-7	IFNγ Treatment of Asynchronous Cells Increases $p21^{WAF1/CIP1}$ Production in (a) T98G, (b) SNB-19, and (c) U-373 Cells	48
3-8	IFNγ Treatment Leads to Increased Binding of $p21^{WAF1/CIP1}$ to Cdk2 in (a) T98G, (b) SNB-19, and (c) U-373 Cells	49
3-9	Antiproliferative Effect of IFNγ on Normal Human Astrocytes	55
3-10	Antiproliferative Effect of ACV on Normal Human Astrocytes	57
3-11	Phosphorylation of [8-^{3}H]-ACV by T98G, SNB-19, U-373, and Normal Human Astrocytes	60

Figure page

3-12 IFNγ and ACV Inhibit Cellular Proliferation of T98G Cells in an Approximately Additive Manner..... 66

3-13 IFNγ and ACV Inhibit Cellular Proliferation of SNB-19 Cells in an Approximately Additive Manner..... 68

3-14 IFNγ and ACV Inhibit Cellular Proliferation of U-373 Cells in an Approximately Additive Manner..... 70

3-15 IFNγ and ACV Inhibit Cellular Proliferation of Normal Human Astrocytes in an Approximately Additive Manner..... 73

3-16 IFNγ Treatment Leads to Increased Binding of $p21^{WAF1/CIP1}$ to PCNA in T98G, SNB-19, and U-373 Cells..... 78

3-17 IFNγ Inhibits Cell Growth in DU145 and PC-3 Cells in a Dose-Dependent Manner..... 81

3-18 IFNγ Upregulates $p21^{WAF1/CIP1}$ Expression in DU145 and PC-3 But Not LNCaP Cells..... 83

3-19 IFNγ Induces STAT1 Phosphorylation in DU145 and PC-3 But Not LNCaP Cells..... 85

3-20 IFNγ Downregulates neu/HER-2 Expression in DU145 and PC-3 Cells... 86

3-21 Downregulation of neu/HER-2 Expression by IFNγ in DU145 and PC-3 Cells is Dose-Dependent..... 87

3-22 Transfection of PC-3 Cells With pRc/RSV-p300 Increases Expression of p300 and Decreases Downregulation of neu/HER-2 in Response to IFNγ Treatment..... 89

3-23 Densitometric Analysis of neu/HER-2 Expression..... 90

3-24 IFNγ Induces STAT1 Phosphorylation in PC-3 (pRc/RSV-p300) Cells in a Dose-Dependent Manner..... 93

3-25 IFNγ Upregulates $p21^{WAF1/CIP1}$ Expression in Wildtype PC-3 and PC-3 (pRc/RSV-p300) Cells..... 95

3-26 Protection of Mice Against Tumor Challenge is Dependent on the Timing of Superantigen Administration Following Vaccination..... 98

Figure page

3-27 Protection of Long Term Survivors Against Rechallenge With Live B16F10 Melanoma Cells..... 99

3-28 Protection of Mice Against Tumor Challenge is Dependent on Dose of Superantigen Administered Following Vaccination..... 102

3-29 Vaccination Followed by Superantigen Administration Results in Increased Tumoricidal Activity..... 106

3-30 Superantigen Administration Results in Increased Serum Levels of IFNγ..... 107

3-31 IFNγ Inhibits Cellular Proliferation of B16F10 Melanoma Cells in a Dose-Dependent Manner In Vitro..... 109

3-32 IFNγ Increases $p21^{WAF1/CIP1}$ Production in B16F10 Melanoma Cells.... 110

3-33 Vaccination Followed by Superantigen Administration Induces a Specific Immune Response..... 112

3-34 Superantigen Administration Prolongs the Survival of Mice With Established Tumor..... 113

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF THE ANTI-TUMOR EFFECTS OF IFNγ AND SUPERANTIGENS IN THE TREATMENT OF CANCER

By

Scott Lee Kominsky

August, 2000

Chair: Howard M. Johnson
Major Department: Microbiology and Cell Science

Interferon (IFN) γ is an important cytokine with a broad range of biological activities affecting various cell types. Superantigens are potent stimulators of T cell activity. I investigated the anti-tumor effects of both IFNγ and superantigens on various cancers, including glioblastoma, prostate cancer, and melanoma. IFNγ directly inhibited the growth of all glioblastomas tested, leading to delays in the cell cycle at the G_1 to S phases. Further, the strength and location of cell cycle delay correlated with IFNγ induction of the cyclin-dependent kinase inhibitor (CKI) $p21^{WAF1/CIP1}$, which bound and inhibited cyclin-dependent kinase 2 (Cdk2). I also showed that IFNγ and acyclovir (ACV) inhibited the glioblastoma cell cycle at the G_1 to S phases in an additive manner. The cooperative antiproliferative effect of IFNγ and ACV on glioblastomas appeared to be due to direct inhibition of DNA synthesis by ACV and IFNγ-induced $p21^{WAF1/CIP1}$ production, which acts by binding

and inhibiting the proliferating cell nuclear antigen (PCNA) and Cdk2. Moreover, the combination of IFNγ and ACV had very little effect on normal human astrocyte cell proliferation.

Consistent with previous findings, IFNγ-induced inhibition of prostate cancer cell line growth was accompanied by an increased production of $p21^{WAF1/CIP1}$, but was also accompanied by the decreased expression of the proto-oncogene neu/HER-2. My findings also implicate the transcriptional co-activator p300 in the positive regulation of neu/HER-2 expression. Further, I propose that IFNγ-induced downregulation of neu/HER-2 may involve the binding of phosphorylated STAT1 to p300 thereby inhibiting neu/HER-2 expression.

Lastly, I found that vaccination with irradiated melanoma cells followed by superantigen administration elicited potent and specific protection of mice against challenge with a syngeneic and poorly immunogenic tumor, resulting in the development of immunological memory. This anti-tumor protection was accompanied by an increase in $CD4^+$ and $CD8^+$ T cell populations, increased CTL activity, and increased serum levels of IFNγ, which may all play a role in mediating this response. In addition, superantigen administration alone prolonged the survival of mice with established tumor. Taken together, these findings suggest a potential role for IFNγ and superantigens in cancer treatment and describe a mechanism for their anti-tumor effects.

CHAPTER 1
INTRODUCTION

Cancer and Cancer Treatment

Cancer is a disease, which can take many forms, affecting numerous people throughout the world each year. It is characterized by unregulated cell growth and can be derived from virtually any cell in the body. Cancer arises due to mutations in the DNA, which are either inherited or accumulated over the course of one's life, resulting in the formation of oncogenes and/or the loss of tumor suppressor genes (Table 1-1). Proto-oncogenes, the precursors to oncogenes, are positive regulators of cell growth. When these genes incur mutations and become oncogenes they are then capable of causing the neoplastic transformation of cells. The genes still control cell proliferation, but are no longer regulated. Tumor suppressor genes, on the other hand, negatively regulate cell proliferation. These genes serve as an intricate system of checks and balances of cell growth in order to prevent tumor formation. When both alleles of these genes become mutated they lose their function and the cell may exhibit uncontrolled proliferation. Considering oncogenes and tumor suppressor genes play a central role in the formation of cancer, a keen understanding of their actions and underlying mechanisms is essential to developing cancer treatments.

It has become readily apparent that the immune system is incapable of eliminating cancers on its own. This may be due to either the tolerization of immune

Table 1-1: Properties of Oncogenes and Tumor Suppressor Genes[a]

Property	Oncogenes	Tumor Suppressor Genes
Mutational events involved	One	Two
Function of mutation	Gain of function	Loss of function
Germ-line inheritance	No	Yes
Effect on growth control	Activate cell growth	Negatively regulate growth-promoting genes
Genetic alterations	Point mutations, gene rearrangements, amplification	Deletions, point mutations

[a]reviewed in Cooper, 1997

system cells to the tumor or the lack of a sufficient response to eradicate the tumor. Currently, cancer treatment is largely limited to surgical removal followed by radiation therapy and/or chemotherapy. This has been effective in the treatment of some cancers, but quite ineffective in the treatment of others. Furthermore, these treatments place a high physical demand on the patient, and can occasionally lead to the patients demise.

Glioblastoma multiforme is one of the most frequently occurring forms of brain cancer (Kleihues et al., 1995). It typically affects adults between the ages of 45 and 60 years and is highly malignant. Glioblastoma originates from astrocytes and is characterized by a loss of cell cycle control frequently involving mutations in tumor suppressor genes such as p53 and p16 (Ohgaki et al., 1993). This leads to rapid cellular division and infiltration of normal brain tissue leading to malfunction of the invaded area as well as an increase in intracranial pressure. Due to the infiltrating nature of these tumors, complete surgical resection is impossible in most cases. Further, current radiation and chemotherapeutic treatments have been shown to extend survival by only several months.

Prostate cancer is the most commonly diagnosed malignancy in men (Parker et al., 1996). It is also the second leading cause of cancer deaths in the Western male population (Bussemakers et al., 1999). Similar to other cancers, prostate cancer arises from a loss or mutation of regulatory factors of the cell cycle such as oncogenes and tumor suppressor genes (Cavenee and White, 1995; Garnick, 1994). Patients with high-grade forms of prostate cancer have a very poor prognosis regardless of available therapies.

Malignant melanoma is an aggressive cancer of the skin originating from melanocytes (Nestle et al., 1998). Incidences of melanoma have been steadily rising year after year and have resulted in rising mortality rates. Due to its invasive nature, melanoma is able to readily metastasize via the lymphatics or blood vessels. Once metastasis has occurred the patient's prognosis is very poor. Thus, early detection and elimination by surgical removal have proven to be the only means of effective management.

In conclusion, glioblastoma multiforme, prostate cancer, and malignant melanoma are dreadful diseases whose incidences are rising. These cancers are responsible for numerous cancer-related deaths each year. Further, current treatments for these diseases are crude and largely ineffective. Therefore, the need for studies into new approaches to the treatment of these cancers is apparent.

Discovery and Biological Activities of IFNγ

Interferons (IFNs) were originally discovered in 1957 by Isaacs and Lindenmann. These proteins were described as inducible cell factors that could transfer antiviral resistance to cells normally susceptible to viral infection. This discovery was made by treating chick chorio-allantoic membrane fragments with heat inactivated influenza virus which resulted in the "interference" of the ability of live influenza virus to replicate in these tissues, hence these cell factors were named interferons (Isaacs and Lindenmann, 1957). Similar to the discovery by Isaacs and Lindenmann, IFNγ was discovered in 1965 by Wheelock due to its antiviral properties. Wheelock reported the induction of an interferon-like viral inhibitor in human leukocytes following phytohemagglutinin treatment (Wheelock, 1965).

Since their discovery, IFNs have been separated into two separate groups based on several factors including physical properties, gene structure, cellular receptor type, cellular source, and biological properties (Table 1-2). IFNs α, β, τ, and ω make up the Type I interferons while IFNγ is the sole member of the Type II interferons. Type I and Type II interferons were found to differ at both the genetic and protein levels (Gray and Goeddel, 1982; Gray and Goeddel, 1983). Further, Type II interferons are solely produced by T lymphocytes and natural killer (NK) cells (Trinchieri and Perussia, 1985). Perhaps the greatest noticeable difference between the Type I and II interferons is in their biological activities. IFNγ has a specific antiviral activity 10-100 times lower than IFNs α and β, but has 100-10,000 times more immunomodulatory activity (Pace et al., 1985).

IFNγ is a cytokine with the ability to induce, stimulate, and repress gene expression leading to antiviral, immunomodulatory, anticellular, and differentiation effects in a variety of cell types (Table 1-3). Although IFNγ was discovered due to its antiviral activity, the IFNγ protein itself does not directly inhibit the multiplication of virus. Instead, IFNγ activates target genes leading to the synthesis of proteins that confer an antiviral state. One of the mechanisms by which IFNγ inhibits virus multiplication is by inhibiting the translation of viral messenger RNA molecules. IFNγ induces the enzyme oligoisoadenylate synthetase, which is responsible for the synthesis of 2,5-OligoA (Zilberstein et al., 1978). 2,5-OligoA in turn activates the ribonuclease Rnase L leading to the cleavage of viral and cellular RNA (Slattery et al., 1979). This creates double stranded RNA, which is responsible for inhibiting protein synthesis. IFNγ treatment may also lead to the establishment of

Table 1-2: Properties of IFNγ[a]

Property	IFNγ
Major Inducers	Antigens and Mitogens
Physical Properties	
M.W. (kDa)	34-50
Amino acids	143
Subunit composition	Noncovalent homodimer
Gene Structure	
Number of genes	1
Chromosomal location	
Murine	10
Human	12
Cellular Source	T cells and NK cells
Biological Properties	Antiviral
	Immunomodulatory
	Modulation of cell differentiation
	Antiproliferative
	Regulation of cell growth
	Regulation of oncogene expression
	Regulation of specific genes

[a]reviewed in Farrar and Schreiber, 1993; Baron et al, 1991; Sen and Lengyel, 1992

Table 1-3: Biological Activities of IFNγ[a]

Antiviral activity
Anticellular activity
Immunoregulatory activities
Facilitation of T helper 1 T-cell stimulation
Inhibition of T helper 2 T-cell stimulation
Activation of cytotoxic T-cells
Activation of natural killer cells
Regulation of antibody production
Stimulation of MHC I and MHC II expression
Activation of macrophages to degrade internalized contents
Stimulation of microbicidal activity of macrophages
Modulation of cell differentiation
Regulation of specific genes

[a]reviewed in Baron et al., 1991; Sen and Lengyel, 1992; Popko et al., 1997

an antiviral state by inhibiting transcriptional events as well as through effects on plasma cell membranes.

IFNγ is probably best known for its immunomodulatory activity. As the primary macrophage activating factor, IFNγ activates tumor cell cytotoxicity, antimicrobial activity, increases killing of intracellular pathogens, and increases the expression of major histocompatibility complex (MHC) expression in macrophages (Pace et al., 1983; Nathan et al., 1983; Torrico et al., 1991; Basham and Merigan, 1983). In addition to its effects on macrophages, IFNγ activates cytotoxic T cells, natural killer (NK) cells, and increases neutrophil mediated phagocytosis and cytotoxicity through the induction of high-affinity IgG F_c receptor expression (Petroni et al., 1988; Perussia et al., 1987). Further, IFNγ plays a role in antibody production, promoting an IgG_{2a} response through a direct effect on B cells (Snapper and Paul, 1987). T cell development and differentiation is also influenced by IFNγ as the presence of IFNγ favors the development of the $CD4^+$ T helper 1 population while suppressing the development of the $CD4^+$ T helper 2 population (Gajewski and Fitch, 1988).

Aside from its antiviral and immunomodulatory effects, IFNγ is able to elicit anticellular effects on a variety of cell types. IFNγ treatment has been shown to inhibit the growth of both normal cells and tumor cells. Further, this inhibition appears to differ depending on the specific tissue type. Several independent studies have shown that the anticellular effects of IFNγ are accompanied by the induction of tumor suppressor gene products or the downregulation of oncogene expression.

Thus, IFNγ has several biological activities, which make it important in the fight against disease.

IFNγ and Disease

IFNγ has proven to be valuable in the treatment of disease due to its antiviral, immunomodulatory, and anticellular activities. In 1990 it was approved by the Food and Drug Administration (FDA) for use in the treatment of chronic granulomatous disease in which granulocytes ingest bacteria, but do not kill them. IFNγ is also being tested in clinical trials for use in the treatment of kidney tumors, basal cell carcinoma, leishmaniasis, Hodgkin's disease, and chronic lymphocytic leukemia (Baron et al., 1991; Johnson et al., 1994; Stuart-Harris et al., 1992). IFNγ is less commonly used in the treatment of viral disease due to the greater antiviral activity of the type I interferons.

IFNγ appears to be most effective in its use as a cancer treatment. The reason for this is twofold. First, IFNγ is able to induce the expression of tumor suppressor genes, which are commonly lost in the development of cancer. IFNγ is also able to downregulate oncogenes, which are overexpressed in many cancers. Second, the strong immunomodulatory activity of IFNγ may play a role in its inhibition of cancer through the activation of cytotoxic T cells, NK cells, and macrophages. The anti-tumor effects of IFNγ may also involve the increased expression of MHC antigens of the surface of cancer cells allowing for their detection and eradication by the immune system.

Although IFNγ is currently being used in the treatment of disease and has tremendous potential in the treatment of others, it is associated with side effects that

restrain its widespread use. IFNγ treatment has been associated with some adverse side effects. Patients receiving IFNγ treatment have reported experiencing flu-like symptoms including fever, headache, chills, fatigue, nausea, vomiting, and diarrhea (Gauci, 1987; Quesada et al., 1986). Other patients have also reported athralgias and myalgias (Spiegel, 1987). These symptoms appear to subside with time and are less severe in patients which were administered smaller doses. Although uncomfortable, these symptoms do not usually necessitate the cessation of treatment and can be controlled through symptomatic regulation of dosage (Vial and Descotes, 1994).

IFNγ and Signal Transduction

Like many other cytokines IFNγ acts through a specific cell surface receptor. This receptor is ubiquitously expressed on all nucleated cells and is species specific (Valente et al., 1992). The IFNγ receptor is composed of two main subunits, the α-chain and the β-chain. The α-chain functions in ligand binding while the β-chain is required primarily in cell signaling. Each receptor chain is associated with a particular Janus kinase (JAK). JAK1 is associated with the α-chain and JAK2 is associated with the β-chain (Igarashi et al., 1994).

Signal transduction begins when IFNγ binds to the α-chains of its receptor as a homodimer (Figure 1-1). This induces the dimerization of the α-chains (Greenlund et al., 1994). Following α-chain dimerization, the β-chains move closer in proximity to the α-chains, which leads to the transphosphorylation and subsequent activation of the associated JAKs. The activated JAKs then phosphorylate specific tyrosine residues on the two receptor α-chains. These phosphorylated tyrosine residues serve as docking sites for signal transducer and activator of transcription 1 (STAT1)

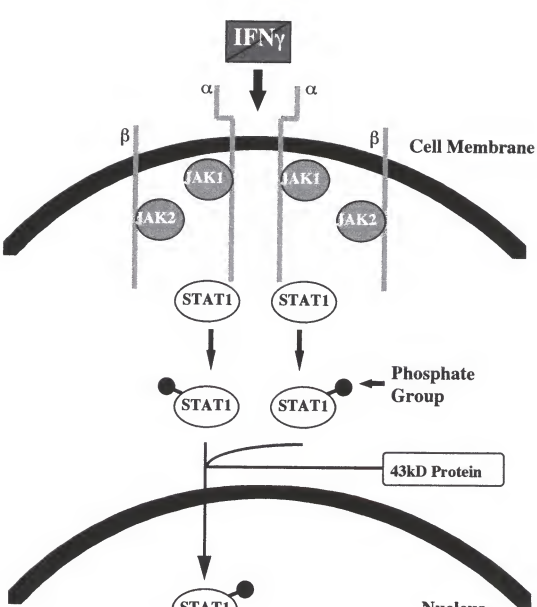


Figure 1-1: The IFNγ Signal Transduction Pathway. IFNγ binds to its receptor as a homodimer and causes the phosphorylation of JAK1 and JAK2. JAK1 then phosphorylates a latent cytoplasmic transcription factor STAT1. Phosphorylated STAT1 dimerizes and translocates to the nucleus where it binds to specific promoter elements known as GAS leading to the transcription of IFNγ-inducible genes.

molecules (Greenlund et al., 1994). Once the STAT1 molecules have bound to the tyrosine residues of the receptor α-chains, they are themselves phosphorylated by the activated JAKs. The phosphorylation of the STAT1 molecules leads to their dissociation from the receptor and the formation of STAT1 homodimers (Greenlund et al., 1995). The complex of phosphorylated STAT1 molecules along with a 43 kilodalton protein is termed the gamma-interferon activating factor or GAF. Following its formation, GAF translocates to the nucleus where it binds to specific DNA sequences termed gamma-activated sites (GAS). This binding of GAF to GAS leads to the transcription of target genes.

Responses to IFNγ are quite rapid due to the speed of the JAK/STAT signal transduction pathway. Following IFNγ binding to its receptor, chromosomal gene transcription is enhanced within 15 minutes without the need for new protein synthesis (Friedman et al., 1984; Larner et al., 1984; Larner et al., 1986; Levy and Darnell, 1990; Reich et al., 1987). The speed of IFNγ signaling is also seen in the rapid turnover of phosphorylated STAT1. Phosphorylated STAT1 levels are negatively regulated by the ubiquitin-proteosome pathway, peaking at 15-30 minutes and decreasing to low levels within 1-2 hours (Kim and Maniatis, 1996). Rapid signal transmission and the apparent lack of second messenger involvement makes the JAK/STAT signal transduction pathway much faster than other pathways.

The Mammalian Cell Cycle and Cancer

The cell cycle is simply the process by which eukaryotic cells replicate. The first concept of a cell cycle was derived from studies performed by Howard and Pelc in 1951. Analyzing ^{32}P-labeled roots of *Vicia faba* seedlings via autoradiography

Howard and Pelc found that DNA was synthesized during a discrete period within the interphase (Howard and Pelc, 1951). Further, they discovered that the period between mitosis and DNA synthesis was quite a bit longer than the period between DNA synthesis and mitosis. This study effectively laid the groundwork for our current model of the cell cycle.

During the cell cycle, the cell proceeds through a series of phases, each to be completed before the next begins. In each phase critical proteins are phosphorylated or dephosphorylated and it is this phosphorylation state that regulates activity, assembly, and cellular localization. The cell cycle is composed of five main phases: G_0, G_1, S, G_2, and M (Figure 1-2). In G_0 the cell is in a state of quiescence. This may occur when there is a depletion of growth factors or when cell crowding occurs. Once the cell has the necessary requirements for growth it enters G_1. In this phase the cell is preparing for DNA synthesis and proteins necessary for this, such as cyclins and cyclin-dependent kinases (Cdks), are produced. While the cell is in the early portion of the G_1 phase, it still has the opportunity to return to G_0 if the conditions for cell replication become unfavorable.

Following the successful completion of G_1 the cell moves into the S phase, in which DNA synthesis occurs. Once the cell enters the S phase it must continue through the completion of DNA synthesis, thus it is very important to ensure that the DNA is not damaged or that any damage has been repaired before entering the S phase. Once DNA synthesis is completed, the cell enters G_2. In this phase the cell is preparing for mitosis once again by producing the necessary proteins. Before leaving G_2, all requirements for mitosis must be completed and any DNA damage

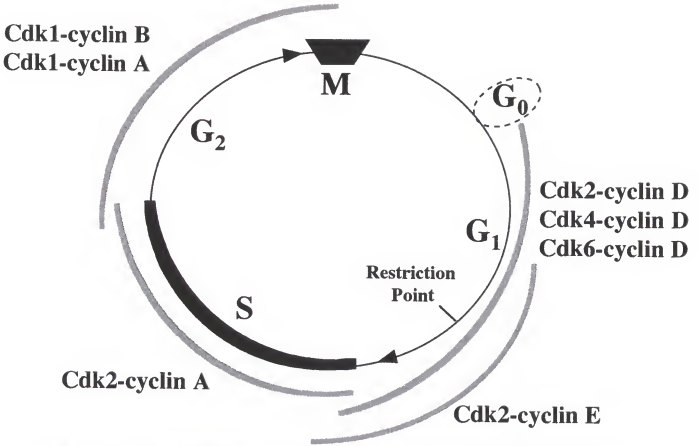


Figure 1-2: The Mammalian Cell Cycle. The cell cycle consists of five phases: G_0, G_1, S, G_2, and M. The Cdk-cyclin complexes that drive the cell cycle are outlined and listed next to each cell cycle phase in which they are active.

occurring during the S phase must be repaired or cell growth arrest will occur. This ensures that damaged or abnormal cells will not be replicated. Finally, the cell enters the M phase or mitosis. In mitosis, the cell is divided into two identical cells. Once the cell enters mitosis it must continue the cycle until it is completed. At this point one round of replication has been completed and the cells return to G_0/G_1.

The mammalian cell cycle is regulated by three main players: transcription factors, cyclins, and cyclin-dependent kinases. Transcription factors are required to initiate or regulate transcription in eukaryotic cells. The most notable transcription factors involved in the mammalian cell cycle belong to the E2F/DP family. E2F and DP family members heterodimerize in various combinations to bind to DNA sequences in the promoter regions of various genes required for the progression from G_1 to S, including c-myc, B-myb, cdc2/Cdk1, cyclin D1, thymidine kinase, DNA polymerase δ, as well as several other genes involved in DNA synthesis and mitosis (Adams and Kaelin, 1995). If E2F is not active, the cell cannot enter the S phase. On the other hand, if E2F is overexpressed, the cell will enter the S phase even in the absence of the necessary requirements. This eventually leads to apoptosis (Beijersbergen and Bernards, 1996). E2F is regulated by the Rb family of proteins, the most notable of which is pRb (retinoblastoma). Rb family members bind E2F and inhibit it from binding and activating promoters of target genes. When pRb is hyperphosphorylated by proteins called cyclin-dependent kinases, it releases E2F allowing it to activate transcription of target genes. Once pRb becomes hypophosphorylated through the action of cell phosphatases it can once again bind to and inhibit E2F.

Cyclins are proteins, which positively regulate the activity of cyclin-dependent kinases. Each Cdk must be complexed with the proper cyclin in order to function. Cyclin levels rise and fall in conjunction with the phases of the cell cycle. As shown in figure 1-2, there are four main families of cyclins involved in driving the cell cycle: cyclins A, B, D, and E. Cyclins D and E are termed G_1 cyclins as they function mostly in the G_1 phase of the cell cycle. Cyclins A and B function in the latter parts of the cell cycle: S, G_2, and M. The cyclins are regulated by proteosomes and are degraded via a ubiquitin-dependent mechanism. Each cyclin contains a structure called a destruction box. The destruction box is made up of short stretches of highly conserved amino acids termed PEST sequences. When the levels of these cyclins need to be lowered, a recognition protein binds the destruction box and the cyclin is then polyubiquinated by a ubiquitin ligase. The polyubiquinated proteins are then rapidly degraded by proteosomes.

Cyclin-dependent kinases are proteins that inactivate (phosphorylate) the retinoblastoma family of proteins. This leads to the release of bound growth-promoting proteins that are necessary for the completion of the cell cycle. There are many different Cdks and each requires the association of a specific cyclin to be active. Cdks are always present in the cell, but since they must be complexed with cyclins to be active and cyclin levels rise and fall, they are only active in certain parts of the cell cycle (Figure 1-2). Each Cdk plays a special role. The Cdk2/4/6-cyclin D complexes are responsible for driving the cell cycle through the G_1 restriction point. Cdk2-cyclin E is the main complex responsible for the activation of DNA transcription factors through the hyperphosphorylation of pRb, which drives

the cell into the S phase. Cdk2-cyclin A then drives the cell through the S phase. Here, Cdk1-cyclin A/B takes over and takes the cell through the G_2/M transition as well as through mitosis, returning the cell to G_0/G_1.

As previously mentioned, the cell cycle is the process by which cells proliferate and cancer arises due to uncontrolled cell proliferation. For this reason, the cell cycle is highly controlled by numerous mechanisms in order to prevent tumor formation. The first mechanism by which the cell cycle is controlled is cyclin degradation. As previously discussed, cyclins contain either PEST sequences or destruction boxes, which allow them to be degraded by proteosomes when their activities are no longer needed. Without this control, Cdks could remain active in phases of the cell cycle in which they are to be inactive. This could lead to errors in DNA synthesis as well as mitosis.

Cyclin-dependent kinases are not solely activated by cyclins, although, they do require cyclins to be active. Cdks are also subject to phosphorylation and dephosphorylation events that play a role in their activation. In yeast, for example, there is a protein named cdc2-activating kinase (CAK), which phosphorylates cdc2 at the threonine-161 residue activating it. The protein Wee1 phosphorylates the tyrosine-15 residue of cdc2 inactivating it. This inactivation occurs even when the threonine-161 residue is phosphorylated. To reactivate cdc2, cdc25 phosphatase removes the phosphate at the tyrosine-15 residue (Baltimore et al, 1995). Cdc25 is a family of phosphatases, each of which is responsible for dephosphorylating certain Cdks when their activity is required in the cell cycle. Some cdc25 proteins are known to form feedback loops with their corresponding Cdk-cyclin complexes

leading to a massive increase in their activity. For example, cdc25C forms a feedback loop with Cdk1-cyclin B. When cdc25C activates the Cdk1-cyclin B complex, Cdk1-cyclin B can then phosphorylate cdc25C, which increases its phosphatase activity. This leads to further activation of Cdk1-cyclin B complexes.

Cdk activity appears to be highly controlled between CAKs and cyclins, but they are further controlled by cyclin-dependent kinase inhibitors (CKIs) (Table 1-4). The main role of the CKIs is to stop Cdks from performing their respective phosphorylation duties leading to a delay or block in cell cycle progression. CKIs can be broken down into two classes. The first class is composed of two main proteins: $p21^{WAF1/CIP1}$ and p27. These CKIs are induced by the tumor suppressor gene p53 (El-Deiry et al., 1993). Interestingly, $p21^{WAF1/CIP1}$ has been shown to be upregulated by p53-independent mechanisms as well, including interferon treatment (Fuse et al., 1996; Hobeika et al., 1997). The $p21^{WAF1/CIP1}$ protein is normally found in Cdk-cyclin complexes, but when the amount of $p21^{WAF1/CIP1}$ bound to these complexes increases the Cdk-cyclin activity is inhibited (Gartel et al., 1996). $p21^{WAF1/CIP1}$ is able to bind most strongly to Cdks complexed with cyclins A, B, D1, and E. $p21^{WAF1/CIP1}$ is also the only CKI that can inhibit Cdk-cyclin A/B complexes (Fuse et al., 1996). The p27 protein, on the other hand, binds strongest to Cdk4 complexes as well as D type cyclins themselves (Hutchinson and Glover, 1995).

The second class of CKIs are termed inactivators of Cdk4 (INK4) proteins and include p16, p15, p18, and p19. These CKIs only bind Cdk4 and Cdk6, the cyclin D dependent kinases, and are less efficient in inhibiting Cdk activity (Hirai et al., 1995). The INK4 proteins exert their effects by competing with the D-type cyclins

Table 1-4: Cyclin-dependent Kinase Inhibitors[a]

Cell cycle phase	Cdk-cyclin complexes	Inhibitors					
		p15	p16	p18	p19	p21	p27
G_1	Cdk4/6-cyclin D	+	+	+	+	+	+/-
G_1/S	Cdk2-cyclin E	-	-	-	-	+	+
S	Cdk2-cyclin A	-	-	-	-	+	-
G_2/M	Cdk1-cyclin B	-	-	-	-	+	-

[a]reviewed in Grana and Reddy, 1995

for the binding of Cdk4 and Cdk6 (Beijersbergen and Bernards, 1996). This leads to the inhibition of the cyclin D dependent kinases preventing the phosphorylation of pRb. Functional failure of these cyclin-dependent kinase inhibitors through mutation can lead to tumor formation (Serrano et al., 1996).

The main means of cell cycle control are checkpoints. Checkpoints are located in critical areas of the cell cycle and serve two very important functions. First, they ensure that the cell has completed all prior phases before moving on to the next. Second, they slow or stop the cell cycle to provide time for the repair of any DNA damage that has occurred. Without these checkpoints cells could incur gene mutations, which could lead to their malignant transformation.

There are two main checkpoints controlling the cell cycle, the first of which is the G_1/S checkpoint. The G_1/S checkpoint is responsible for stopping the cell cycle in response to DNA damage. When DNA damage occurs the tumor suppressor gene p53 is activated and the levels of p53 protein increase (Hutchinson and Glover, 1995). These increased levels of p53 lead to the transcription of the CKI $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ then binds to Cdk2-cyclin E and inhibits the phosphorylation of pRb, preventing the release of E2F. This chain of events leads to cell cycle arrest until the DNA damage is repaired.

The G_2/M checkpoint is another main player in controlling the cell cycle. It functions in stopping the cell cycle when DNA damage occurs and in preventing the onset of mitosis when DNA synthesis has not been completed, protecting against mitotic errors. The G_2/M checkpoint is able to affect a number of cell cycle players, but the exact mechanism of its action has not been elucidated. During a DNA

damage induced G_2 delay, the levels of p53 rise, the activity of Cdk1 is inhibited, and the phosphorylation events of cdc25C are suppressed (Kaufman and Paules, 1996). If DNA damage is severe at either checkpoint, the tumor suppressor p53 can induce apoptosis, ensuring that a malfunctioning cell does not become dangerous to the body.

Even though the cell cycle is tightly regulated by many different mechanisms, cancers still arise due to uncontrolled cell cycling. Generally, these cancers do not result from a single mutational event, but rather from an accumulation of several mutations in regulatory genes such as tumor suppressor genes. Three tumor suppressor genes frequently found mutated in various cancers are pRb, p16, and p53. Mutation of pRb is well known for causing the eye disease retinoblastoma. Rb is a family of proteins that also includes p107 and p130. All proteins in this family serve in binding the E2F family of transcription factors, thus preventing them from activating transcription (Sala et al., 1996). Defects in the p107 and p130 proteins have not been found in human cancer, but a defective pRb gene has been found to play a role in the development of lung, bladder, and pancreatic cancers.

p16 is a more recently characterized tumor suppressor gene. Many tumor derived cell lines have been found to have a deletion of this gene. Further, when the p16 gene is placed into lung cancer cell lines deficient in p16, tumor cell proliferation is suppressed both *in vitro* and *in vivo* (Jin et al., 1995). p16 is able to inhibit cell growth through the binding and inhibiting of Cdk4 and Cdk6, preventing the tumor suppressor pRb from releasing E2F and subsequently activating transcription. Thus, the growth suppressive effects of p16 are dependent on a

wildtype pRb protein and are not seen in cell lines lacking a wildtype pRb (Medema et al., 1995).

The tumor suppressor p53 is a well-studied gene, which has been found to play an important role in protecting against tumor formation (Kastan et al., 1991). It is frequently found mutated or deleted in the majority of human tumor types studied. p53 guards against tumor formation in two main ways (Figure 1-3). First, it can signal the cell to undergo apoptosis. Second, it can slow or stop the cell cycle in order to create time for the repair of damaged DNA. The tumor suppressor p53 plays a central role at both the G_1/S and G_2/M checkpoints. Without a wildtype p53 these checkpoints will fail and cells may be replicated even after DNA damage. However, p53 is not responsible for inhibiting the progression of the cell cycle on its own. In the event of DNA damage, p53 levels rise, leading to the transcriptional activation of several genes encoding CKIs such as $p21^{WAF1/CIP1}$ and p27, which then inhibit the progression of the cell cycle.

The main transcriptional target of p53, $p21^{WAF1/CIP1}$, is arguably one of the most important CKIs in the defense against uncontrolled cell cycling, and thus tumor formation. Its production is not solely dependent on p53, as it has been shown to be produced by p53-independent mechanisms, including interferon treatment (Jiang et al., 1994; Parker et al., 1996). It has been shown that STAT1, which is activated following treatment of cells with IFNγ, binds to the promoter region of the $p21^{WAF1/CIP1}$ gene and induces transcription (Chin et al., 1996). $p21^{WAF1/CIP1}$ is a multifaceted protein capable of two main interactions that lead to the inhibition of cell growth (Xiong et al., 1993; Waga et al., 1994). As previously mentioned,

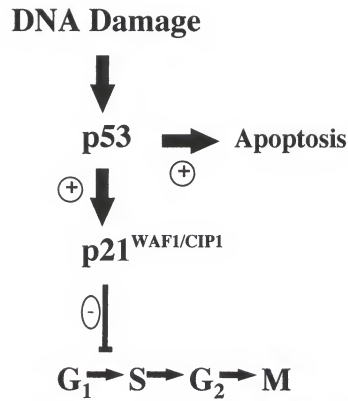


Figure 1-3: The Role of p53 in Guarding Against Tumor Formation. DNA damage induces p53 expression, which may lead to either apoptosis or the induction of $p21^{WAF1/CIP1}$. The induction of $p21^{WAF1/CIP1}$ results in the inhibition of Cdk-cyclin complexes resulting in a delay in cell cycle progression allowing time for DNA repair before DNA replication in the S phase.

$p21^{WAF1/CIP1}$ is able to bind and inhibit most Cdk-cyclin complexes. It is also able to inhibit DNA replication by binding to the proliferating-cell nuclear antigen (PCNA), preventing it from activating DNA polymerase δ. $p21^{WAF1/CIP1}$ has been shown to be able to interact with both Cdks and PCNA on an individual basis, but can bind them simultaneously as well (Gartel et al., 1996). A number of tumor cell lines have been shown to undergo cell cycle arrest in the G_1 phase after incorporation of $p21^{WAF1/CIP1}$ constructs. Further, the overexpression of $p21^{WAF1/CIP1}$ in a melanoma cell line was shown to inhibit growth via an accumulation of cells in the G_0/G_1 phase of the cell cycle (Gartel et al., 1996). Thus, $p21^{WAF1/CIP1}$ is a potent inhibitor of the cell cycle and may play an important role in the prevention and treatment of cancer.

Nucleoside Analogs and Disease

Nucleic acids are an essential component of all living organisms. They are synthesized by polymerases, which piece them together as strands of individual nucleotides. Nucleoside analogs are molecules that mimic the natural substrates of DNA polymerases (adenosine, guanosine, cytidine, or thymidine), but are slightly different in structure such that additional nucleotides cannot be added following their incorporation into a growing DNA strand. For example, the nucleoside analog acyclovir (ACV), an analog of guanosine, lacks a free 3′-OH onto which subsequent nucleotides are added during DNA synthesis. Thus, when ACV is incorporated into a growing DNA strand it acts as a mandatory DNA chain terminator and can severely inhibit the cell cycle. Nucleoside analogs may also irreversibly bind to DNA polymerases. Each of these actions leads to the disruption of DNA synthesis, and thus inhibits cellular proliferation.

Nucleoside analogs, such as ACV, are currently being used in the treatment of several viral diseases. They are also being tested in combination with IFN in the treatment of herpesviruses (Hanada et al., 1989). Nucleoside analogs have been popular in the treatment of viral diseases due to their "specificity" against virally infected cells. In order to become active in the cell, nucleoside analogs must first be converted to their triphosphate form. It has been suggested that the nucleoside analog acyclovir can only be phosphorylated by the viral thymidine kinase. However, it has been shown that guanosine can also be phosphorylated by cellular deoxyguanosine and deoxycytidine kinases albeit with less efficiency than thymidine kinase (Kawasaki et al., 1992; Sarup and Fridland, 1987; Sjoberg et al., 1998). In fact, previous studies have suggested that the thymidine kinases of herpesviruses may have evolved from cellular deoxycytidine kinases (Harrison et al., 1991).

Due to their ability to inhibit cellular proliferation, nucleoside analogs are currently being tested in the treatment of several cancers (Steinmetz et al., 1999). In a recent study, ACV was shown to have an inhibitory effect on leukemia cell growth (Nishimaki et al., 1996). Interestingly, the expression of deoxycytidine kinase has been found to be increased in malignant cells, possibly contributing to the antiproliferative effects of nucleoside analogs in various cancer cell types (Spasokoukotskaja et al., 1995). Thus, nucleoside analogs may play a significant role in the treatment of cancer.

Growth Factor Receptors and Transcriptional Co-activators

Growth factors are proteins that stimulate cell proliferation. Many growth factors have been discovered, which can affect the growth of virtually all cell types.

These proteins stimulate growth by binding to their corresponding receptors on the cell surface, generating a signal for the cell to grow. Not surprisingly, growth factors have been implicated in the development of cancer as they have been found to influence constitutive activation of growth promoting pathways in cancer cells (Aaronson, 1991). Several oncogenes have been found to encode growth factors and tyrosine kinase receptors that participate in mitogenic signaling (Bishop, 1991). There is much evidence for genetic aberrations affecting both growth factors and their receptors in human malignancies.

Among growth factor receptors, members of the epidermal growth factor receptor family (EGFR) are most frequently implicated in human cancer (Aaronson, 1991). One member of this family is HER-2, which is a tyrosine kinase receptor encoded by the proto-oncogene neu/HER-2. The neu/HER-2 proto-oncogene has been linked to several human malignancies including breast, ovarian, and prostate cancer (Slamon et al., 1987; Slamon et al., 1989; Moul et al., 1992). Previous studies have shown that the overexpression of neu/HER-2 alone is sufficient to cause malignant transformation (Guy et al., 1992).

Transcriptional co-activators are proteins that potentiate the activity of transcription factors by acting as adaptors that bridge enhancer-specific factors to the basal transcriptional machinery, resulting in the transactivation of target genes. The CBP/p300 family of proteins is a group of transcriptional co-activators that can interact with a variety of DNA-binding factors (Janknecht and Hunter, 1996). p300 was originally isolated and cloned due to its interaction with the E1A protein of adenovirus (Eckner et al., 1994). E1A does not directly bind DNA, but regulates

gene expression via interactions with multiple transcription factors (Dyson and Harlow, 1992). As a result, E1A is responsible for reducing p300 co-activation of several genes controlling activities such as cellular differentiation, cell cycle progression, and transformation (Giordano and Avantaggiati, 1999).

Recently, it has been shown that neu/HER-2 promotor activity can be repressed by the E1A protein of adenovirus (Yu et al., 1990). Further, this repression was reversed both by increasing amounts of p300 as well as by normal amounts of p300 defective in E1A binding (Chen and Hung, 1997). These findings suggest that the transcriptional co-activator p300 may play a role in the regulation of the neu/HER-2 gene. Interestingly, phosphorylated STAT1 homodimers from IFNγ-treated cell extracts have also been shown to bind the E1A binding site of p300, indicating the potential for IFNγ to repress p300 regulated genes via phosphorylated STAT1 (Zhang et al., 1996).

Discovery and Biological Activity of Superantigens

Superantigens are proteins that form complexes with major histocompatibility complex (MHC) class two molecules on antigen presenting cells (APCs), binding to the outside of the antigen-binding cleft, and stimulating T cells via V_β-specific elements of the T cell receptor (Mollick et al., 1989). Superantigens were originally discovered in the early 1970s in the form of minor lymphocyte stimulating (Mls) determinants in mice. These Mls determinants were found to be capable of stimulating a much higher number of T cells than any particular foreign antigen in MHC-identical mouse strain combinations as measured by mixed lymphocyte reaction (Janeway et al., 1980). Mls determinants are termed self-superantigens as

they are found naturally in mice, however numerous foreign superantigens exist as well. Foreign superantigens are produced by bacteria such as *Staphylococcus aureus,* Group A streptococci, and *Mycoplasma arthritidis.* They have also been shown to be produced by several viruses including mouse mammary tumor virus, mouse leukemia virus, and human spumaretrovirus. Further these superantigens appear to play a role in several diseases, which include food poisoning, toxic shock syndrome, arthritis, and mammary tumors (Bergdoll, 1985; Cole and Griffiths, 1993; Held et al., 1994).

The staphylococcal enterotoxins (SEs) are the classical models of superantigens and are responsible for food poisoning as well as toxic shock syndrome. This family of superantigens includes SEA, SEB, SEC1-3, SED, and SEE. The biological activity of these enterotoxins has been studied for many years and studies elucidating the mechanism of their actions are ongoing. Superantigens are unique in their presentation by APCs in several respects. First, as opposed to conventional antigens, superantigens are not processed. Second, superantigens bind to the outside of the MHC class II binding cleft, whereas conventional antigens are MHC restricted and bind on the inside of the binding cleft. Lastly, the binding of each superantigen to T cells is restricted to a specific V_β region of the T cell receptor.

Superantigens are considered to be T cell mitogens as the unique interaction of superantigens with both APCs and T cells produces a signal that may lead to the activation of as much as 20% of the T cell repertoire. Both $CD4^+$ and $CD8^+$ T cells proliferate in response to the SEs (Fleischer and Schrezenmeier, 1988). This massive activation of T cells is accompanied by a tremendous release of cytokines,

including IFNγ and tumor necrosis factor (TNF) α. These cytokines start a signaling cascade that can result in the production of inflammatory mediators, such as interleukins (IL) 1 and 6. Ultimately, it is the activity of these cytokines that is responsible for the onset of food poisoning and toxic shock syndrome. The hyper-activation of T cells generally occurs within 48 hours following SE exposure. Thereafter, it is widely held that the activated T cells undergo clonal deletion or are led to a state of functional anergy. This process is beneficial to the superantigen-producing bacteria as it leads to a state of immunosuppression in the host.

Although superantigens are associated with several human diseases, they may have promise in treating disease as well. The immense and rapid proliferation of T cells may be beneficial if directed at a pathogen. A recent study showed that SEB treatment enhanced the resolution of an induced urinary tract infection (Morin and Hopkins, 1998). Further, studies have shown that T cell clonal expansion can be furthered following rechallenge with SEA and that T cells can be rescued from SEA-induced anergy (Aoki and Yoshikai, 1997; Belfrage et al., 1997). If the power of superantigens could be harnessed, they could prove to be very effective in immunotherapy. Their ability to greatly amplify T cell responses would be especially beneficial in the treatment of diseases such as cancer, where a very small and ineffective response is typically generated.

CHAPTER 2
MATERIALS AND METHODS

Reagents, Cell Lines, and Plasmids

Purified human IFNγ (specific activity 1 x 10^7 units/mg) was obtained from Genzyme (Cambridge, MA, USA). Acycloguanosine (ACV) was obtained from Sigma Co (St. Louis, MO, USA). Transfectam reagent was obtained from Promega (Madison, WI, USA). T98G, U-373, SNB-19, DU145, LNCaP, PC-3, B16F10, and Lewis Lung carcinoma (LL/2) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Normal human astrocytes were obtained from Clonetics Corporation (Walkersville, MD, USA). Complete media for T98G cells consisted of Eagles minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin, and 200 μg/ml streptomycin. Complete media for U-373 cells consisted of EMEM supplemented with 10% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. Complete media for SNB-19 cells consisted of Ham's F10 medium supplemented with 15% FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin. Complete media for the DU145, LNCaP, and PC-3 cell lines consisted of EMEM, EMEM + 1 mM sodium pyruvate and 2 mM L-glutamate, and F12K medium, respectively, supplemented with 10% FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin. Complete media for B16F10 cells

consisted of EMEM + BME Vitamin Solution (Sigma Co., St. Louis, MO, USA) supplemented with 10% FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin. Complete media for LL/2 cells consisted of Dulbecco's modified eagles medium (DMEM) supplemented with 10% FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin. Complete media for normal human astrocytes consisted of Astrocyte Growth Medium Bulletkit as supplied by Clonetics Corporation. Starvation media for cell synchronization consisted of the appropriate above media supplemented with 0.5% FBS. The superantigens SEA and SEB were obtained from Toxin Technology (Sarasota, FL, USA). Antibodies to $p21^{WAF1/CIP1}$, PCNA, STAT1, and p300 were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antibodies to phosphorylated STAT1 were obtained from New England BioLabs, Inc (Beverly, MA, USA). Antibodies to neu/HER-2 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies to $CD4^+$, $CD8^+$, and NK were obtained from PharMingen (San Diego, CA, USA). The p300 plasmid pRc/RSV-p300 was generously provided by Dr. Richard H. Goodman (Vollum Institute, Oregon Health Sciences University, Portland, OR).

Antiproliferation Assay

Inhibition of cell growth was determined by plating cells at 4-10 x 10^4 cells/well in six well plates. The cells were grown in complete media for 24 hours and subsequently treated with IFNγ, ACV, or both at various concentrations, versus media alone. The cells were trypsinized and removed from the plates at 24-96 hours. Cells were then counted using a hemocytometer and cell viability was determined by trypan blue dye (0.4%) exclusion.

Cell Cycle Analysis

T98G, U-373, and SNB-19 cells were seeded into 25 cm^2 flasks (Sarstedt, Newton, NC, USA) and synchronized in the G_0/G_1 phase of the cell cycle by culturing in starvation media for 24 (T98G) or 48 (U-373 and SNB-19) hours. Cells were then placed in complete media and subsequently treated with IFNγ, ACV, or both at various concentrations, versus media alone. At appropriate time points cells were harvested and washed twice in sample buffer (PBS containing 1% glucose). After centrifugation, cells were resuspended in 1 ml sample buffer, centrifuged and fixed by adding 1 ml cold ethanol (-20°C) dropwise while gently vortexing. Samples were then stored at 4°C for at least 24 hours. Prior to analysis, cells were washed twice with sample buffer and resuspended in 600-1000 µl sample buffer containing 50 µg/ml propidium iodide and 50 units/ml Rnase A (Sigma Co, St. Louis, MO, USA). Samples were covered with foil and allowed to stain for up to 2 hours at room temperature. Samples were then filtered through 44 µm nylon mesh and analyzed using a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and the LYSYS II software system. Cell cycle data was then analyzed using either CellFIT or ModFIT software.

CTL Assay

Mice were vaccinated i.p. with 1 x 10^6 irradiated (15000 rads) B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 µg each SEA and SEB at day 6. Spleens were removed and T cells were isolated using the Cellect-plus mouse T cell kit (Biotex Laboratories, Alberta). B16F10 target cells were labeled with 300 µCi ^{51}Cr (Amersham, Arlington Heights, IL, USA) and plated in 96

well plates at 10^5 cells/well. T cells were washed, counted, and added to labeled B16F10 cells as effectors for 4 hours at 37°C. Plates were centrifuged, supernatants were collected, and the amount of released ^{51}Cr was detected via gamma counter (LKB-Wallac, Turku 10, Finland).

ELISA for IFNγ

Mice were injected i.p. with 25 μg each SEA and SEB. Serum samples were subsequently collected over a period of 48 hours. Samples were diluted and then assayed for IFNγ concentration by ELISA using Cytoscreen for mouse IFNγ (Biosource, Camarillo, CA, USA). Color development was monitored at 450 nm in an ELISA plate reader (Bio-Rad, Richmond, CA, USA).

Expansion of $CD4^+$, $CD8^+$, and NK Cells

C57BL/6 female mice (8-12 weeks old) were vaccinated i.p. with 1 x 10^6 irradiated (15000 rads) B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged i.p. with 2.5 x 10^5 live B16F10 cells at day 13. For analysis of $CD4^+$, $CD8^+$, and NK cell expansion, spleen cells were obtained from mice in each of the treatment groups at day 13 and day 27. Cells (1 x 10^6) were washed with FACS buffer (PBS containing 0.5% bovine serum albumin and 10 mM sodium azide) and then incubated with fluorescein isothiocyanate-labeled anti-$CD4^+$, $CD8^+$, and NK antibodies for 30 minutes at 4°C. Cells were then washed in FACS buffer and

analyzed on a FACScalibur (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Immunoprecipitation and Immunoblotting

For preparation of cell lysates, cells (4-6 x 10^6 cells/sample) were grown in the presence of absence of various concentrations of IFNγ for various lengths of time and lysed at 4°C for 20 minutes in 500 µl of cold lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.25 M NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 10% (vol/vol) glycerol, 0.1% (vol/vol) Nonidet P-40, 2 mM Na-orthovanadate, 2 mM dithiothreitol, leupeptin (10 µg/ml), pepstatin (10 µg/ml), aprotinin (10 µg/ml), benzamidine (5 µg/ml), and 1 mM phenylmethanesulfonyl fluoride. Equal amounts of protein from cell lysates were immunoprecipitated with 1 µg of antibody specific for protein of interest and resolved by SDS-PAGE (Biorad, Hercules, CA, USA). Protein was then transferred to ECL nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). Following Western transfer, membranes were immunoblotted with antibodies specific for the proteins of interest and developed using ECL (Amersham, Arlington Heights, IL, USA). Densitometric analysis of radiographic film using IA-200 Image Analysis Software was used to determine percent decrease or fold-increase between band intensities based on total pixel values.

In Vitro Kinase Assays

Cyclin-dependent kinase activity was measured by immunoprecipitation of Cdk2 as described above. Following immunoprecipitation, samples were washed

three times with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.5, containing 1 mM EGTA, 10 mM $MgCl_2$, 1 mM Na-orthovanadate, 2 mM DTT, 5 mM ATP, 20 mM β-glyceryl phosphate) and incubated in kinase buffer containing 5 mg histone H1 and 20 mCi of [^{32}P] γ-ATP (specific activity 6000 Ci/mmol; 1 mCi = 37 Mbq) (Amersham, Arlington Heights, IL, USA) in a final volume of 30 μl at 30°C for 10 minutes. Samples were then centrifuged and analyzed for histone H1 phosphorylation by a filter-binding assay using centrifugal Pierce phosphocellulose units, SpinZyme™ Format purchased from Pierce (Rockford, IL, USA) according to the manufacturer's instructions.

Phosphorylation of ACV was measured in a reaction containing: equal amounts of protein from cell lysates, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 10 mM NaF, and [8-^{3}H]-ACV (Moravek Biochemicals, Brea, CA, USA) in a total volume of 100 μl. Samples were incubated at 37°C for 30 minutes. 25 μl of each sample was spotted onto DE-81 filter disks (Whatman) and subsequently washed three times with 5 ml dH_2O in a 1225 sampling manifold (Millipore, Bedford, MA, USA). Filter disks were then placed in scintillation vials containing 1 ml of 0.2 M KCl in 0.1 N HCl for 15 minutes to elute phosphorylated [8-^{3}H]-ACV. Scintillation fluid was added and radioactivity was counted via scintillation counter (Beckman LS 3801, USA).

In Vivo Vaccination: Tumor Challenge Experiments

C57BL/6 female mice (8-12 weeks old; The Jackson Laboratory) were vaccinated i.p. with 1 x 10^6 irradiated (15000 rads) B16F10 melanoma cells (derived from C57BL/6) at day 0 and subsequently injected i.p. with either 6.25, 12.5, or 25

μg each SEA and SEB at either day 6 or day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged with either 2.5 x 10^5 live B16F10 cells or 1 x 10^6 LL/2 cells at day 13. Mice were evaluated on a daily basis and sacrificed when moribund.

For rechallenge experiments, C57BL/6 female mice surviving tumor challenge for over 150 days were injected with 1 x 10^4 live B16F10 cells. Naïve mice served as control. Mice were evaluated on a daily basis and sacrificed when moribund.

For treatment of established tumor, C57BL/6 female mice were injected i.p. with 1 x 10^4 live B16F10 melanoma cells at day 0 and subsequently injected with 25 μg each SEA and SEB at day 6. Mice were evaluated on a daily basis and sacrificed when moribund.

Transfection

PC-3 cells were transfected with the plasmids pRc/RSV and pRc/RSV-p300 following the Transfectam® reagent transfection protocol. PC-3 cells (1.3 x 10^5) were plated in a 100 mm^2 plate. A mixture of either pRc/RSV or pRc/RSV-p300 DNA and Transfectam® reagent was added to the PC-3 cells and left in contact for 2 hours. Transfected PC-3 cells were then selected via incubation with complete media + 600 μg/ml geneticin (Sigma Co, St. Louis, MO, USA).

CHAPTER 3
RESULTS

IFNγ Inhibition of the Glioblastoma Cell Cycle

Inhibition of Cell Growth

We began our study by determining the antiproliferative effects of the type II IFN, IFNγ, on the human glioblastoma cell lines T98G, SNB-19, and U-373. These cells were treated with IFNγ at concentrations ranging from 625-10000 units/ml and compared to media-treated cells for inhibition of growth as determined by direct cell counts. IFNγ inhibited the growth of all cell lines in a dose dependent manner, with T98G and SNB-19 cells being most susceptible (Figure 3-1). Dose-response data indicated that 2500 units/ml of IFNγ was the lowest concentration that achieved maximal inhibition, and this concentration was used in subsequent experiments.

The cell lines were then treated with IFNγ, and direct cell counts were obtained at different days of culture (Table 3-1). As was found in the dose-response studies, IFNγ was able to inhibit the growth of all cell lines to varying degrees, with the greatest effect on cell lines T98G (82.2% inhibition) and SNB-19 (63.4% inhibition). The cell line U-373 was the least inhibited by IFNγ at 45.4% inhibition. The percent viability of all of the IFNγ-treated cell lines was approximately 92%, suggesting that the mechanism of inhibition was not cytotoxicity or induced cell death. Thus, IFNγ inhibited cell growth of several glioblastoma cell lines, but to varying degrees.

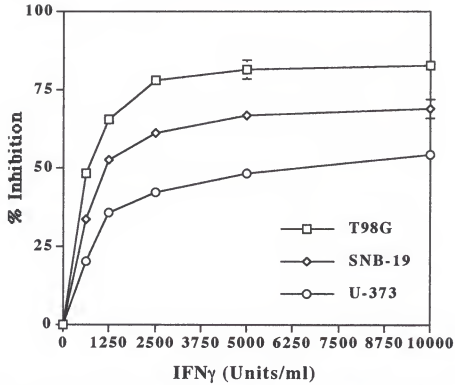


Figure 3-1: IFNγ Inhibits Cellular Proliferation of Glioblastoma Cell Lines T98G, SNB-19, and U-373 In a Dose-Dependent Manner. Cells (1×10^5 cells/well) were incubated with or without IFNγ at concentrations of 625, 1250, 2500, 5000, and 10000 units/ml for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability for all cell lines was approximately 92%. Data from three representative experiments are expressed as total number of cells per sample ± S.D.

Table 3-1: Inhibition of Glioblastoma Cell Line Growth[a]

	% Inhibition		
Time (hrs)	T98G	SNB-19	U-373
48	53.1	28.3	12.5
72	56.0	57.3	4.0
96	82.2	63.4	45.4

[a]Data are from three representative experiments performed in triplicate. Percent inhibition values were calculated directly from cell count experiments using 2500 units/ml IFN_{γ}.

Cell Cycle Analysis

We next determined the effects of IFNγ on the cell cycle of glioblastoma cell lines using flow cytometry in order to determine the cell cycle events affected by IFNγ. Glioblastoma cells were synchronized into G_0/G_1 by serum starvation in order to facilitate observation of events at the various stages of the cell cycle. Cells were then stimulated to grow by addition of serum in the presence or absence of IFNγ. T98G cells showed a very strong G_1 delay with 77.9% of IFNγ-treated cells still in the G_1 phase at 16 hours versus 48.4% of untreated cells (Figure 3-2). By 24 hours, untreated cells had almost completed one full cycle while the IFNγ-treated cells were just entering the S phase (36.7%). This strong delay in the cell cycle correlated with the strong antiproliferative effect of IFNγ seen on these cells in direct cell counts (Figure 3-1). U-373 cells showed a moderate G_1 delay, which correlated with the moderate antiproliferative effects of IFNγ on this cell line (Figure 3-3). At 12 hours, 79.2% of the IFNγ-treated cells were in the G_1 phase of the cell cycle compared to 72.1% of the untreated cells. This delay was then carried through a full cell cycle. SNB-19 cells showed a moderate G_1 delay at 20 hours with 52.3% of the IFNγ-treated cells in the G_1 phase versus 40.8% of the untreated cells (Figure 3-4). Unlike the predominant G_1 effects seen in the other cell lines, this delay became more pronounced in the S phase. This was seen at 32 hours where the untreated cells had already completed one cell cycle versus the IFNγ-treated cells, which were still in the S phase at 51.5%. This strong delay also correlated with the strong antiproliferative effect of IFNγ on this cell line. Thus, IFNγ inhibited glioblastoma cell proliferation to varying degrees, and at different stages of the cell cycle.

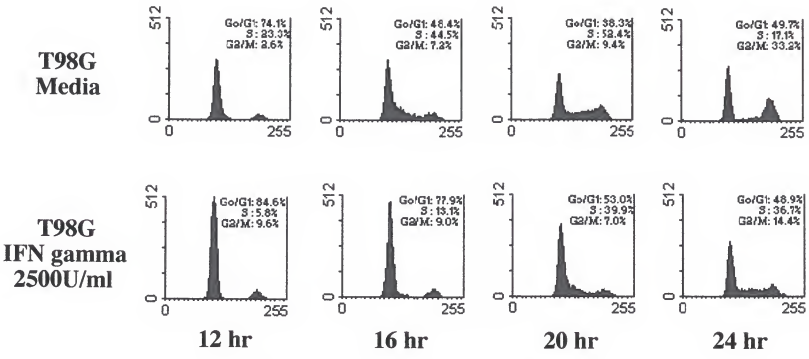


Figure 3-2: IFNγ Inhibits the Progression of T98G Cells Through the Cell Cycle Causing a Strong G_1 Delay. T98G cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis. Horizontal axis: relative fluorescence intensity. Vertical axis: number of cells.

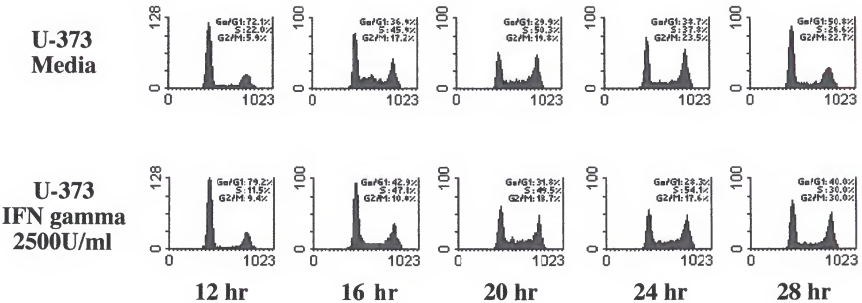


Figure 3-3: IFNγ Inhibits the Progression of U-373 Cells Through the Cell Cycle Causing a G_1 Delay. U-373 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis. Horizontal axis: relative fluorescence intensity. Vertical axis: number of cells.

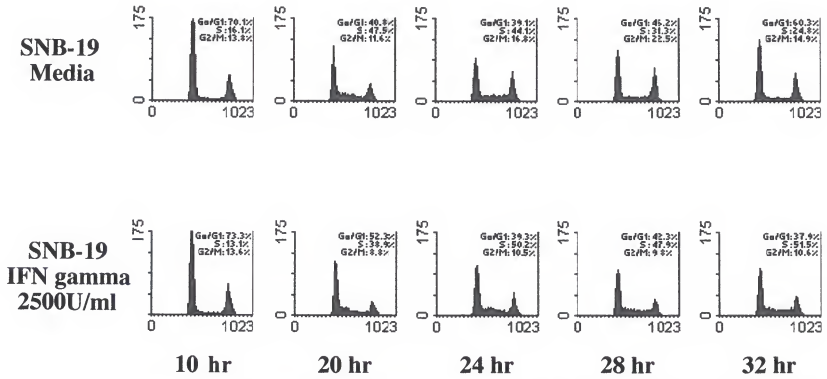


Figure 3-4: IFNγ Inhibits the Progression of SNB-19 Cells Through the Cell Cycle Causing a Strong G_1-S Delay. SNB-19 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis. Horizontal axis: relative fluorescence intensity. Vertical axis: number of cells.

Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$

To examine the mechanism involved in the antiproliferative effects of IFNγ on these glioblastoma cell lines, we looked at the production of the CKI, $p21^{WAF1/CIP1}$, in response to IFNγ treatment. Synchronous cells from each cell line were treated with IFNγ or media for 4-16 hours. Immunoblot analysis using anti-$p21^{WAF1/CIP1}$ antibody on cell lysates showed increased $p21^{WAF1/CIP1}$ production in IFNγ-treated cells as compared to untreated cells (Figure 3-5). Cells that were more sensitive to IFNγ antiproliferative activity showed greater increases in $p21^{WAF1/CIP1}$ production at 8 hours in response to IFNγ treatment relative to untreated cells (Figure 3-6).

T98G cells showed a 73-fold relative increase in $p21^{WAF1/CIP1}$ production when compared to control, which was the highest of all cell lines tested as measured by densitometric scanning. This result correlated with the fact that T98G cells are also the most inhibited in the G_1 phase of the cell cycle by IFNγ treatment (Figure 3-2). IFNγ-treated T98G cells exhibited the most elevated $p21^{WAF1/CIP1}$ levels at 4 and 8 hours compared to untreated cells. These results are consistent with the strong G_1 delay observed in these cells by flow cytometry. SNB-19 cells showed the second largest increase in $p21^{WAF1/CIP1}$ production in response to IFNγ at 15.5-fold and were also the second most inhibited by IFNγ treatment. U-373 cells showed a much lower increase in $p21^{WAF1/CIP1}$ production, 2.6-fold, as compared to T98G and SNB-19 cells. This is consistent with the reduced sensitivity to IFNγ induced growth inhibition and with the moderate early G_1 delay seen in this cell line. Thus, sensitivity to IFNγ antiproliferative activity correlated with the increased levels of

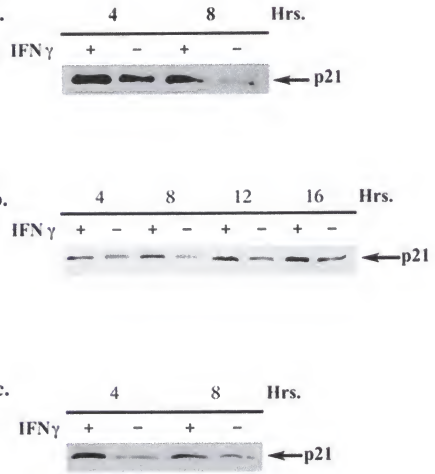


Figure 3-5: IFNγ Treatment of Synchronized Cells (by serum starvation) Increases $p21^{WAF1/CIP1}$ Production in (a) T98G, (b) SNB-19, and (c) U-373 Cells. Cells were grown for 24 hours in starvation media and subsequently incubated from 4-16 hours in complete media with or without 2500 units/ml IFNγ. Immunoblot analysis was performed on cell lysates using anti-$p21^{WAF1/CIP1}$ antibodies as described in Materials and Methods. $p21^{WAF1/CIP1}$ levels in T98G and U-373 IFNγ-treated cells dropped to those seen in untreated cells at both 12 and 16 hours (data not shown).

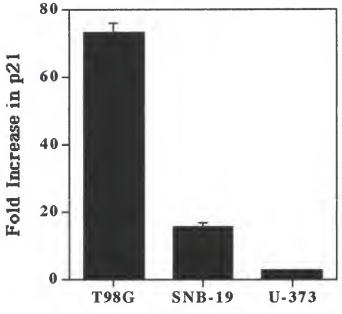


Figure 3-6: IFNγ Treatment Inreases $p21^{WAF1/CIP1}$ Production. The fold increase in $p21^{WAF1/CIP1}$ levels of IFNγ-treated cells compared to untreated cells at 8 hrs. was determined by densitometric scanning of radiographic film for all cell lines.

$p21^{WAF1/CIP1}$ following IFNγ treatment in these cell lines, with the greatest increases in $p21^{WAF1/CIP1}$ production in those cell lines that were most inhibited by IFNγ.

Asynchronous cells were next tested for the production of $p21^{WAF1/CIP1}$ in response to IFNγ, since serum starvation itself causes some increased levels of $p21^{WAF1/CIP1}$. Each cell line was treated with or without IFNγ in complete media for 4-16 hours and $p21^{WAF1/CIP1}$ levels were assessed as described above. IFNγ-treated cells showed elevated levels of $p21^{WAF1/CIP1}$ compared to untreated cells (Figure 3-7), indicating that IFNγ treatment increases levels of $p21^{WAF1/CIP1}$. It should be noted, however, that throughout this study synchronous cells were used to better follow cell cycle events.

To determine the ability of IFNγ to increase the production of other CKIs we immunoblotted for the CKIs p16 and p27. IFNγ treatment did not result in increased levels of p16 or p27 in any of the cell lines (data not shown). Thus, IFNγ inhibitory effects do not involve p16 or p27.

Induction of $p21^{WAF1/CIP1}$ Leads to Increased Binding of $p21^{WAF1/CIP1}$ to Cdk2

To determine if $p21^{WAF1/CIP1}$ had a direct effect on the cell cycle we looked at the binding of $p21^{WAF1/CIP1}$ to Cdk2, which is active in the G_1 and S phases of the cell cycle. T98G, SNB-19, and U-373 cells were synchronized by serum starvation and subsequently treated with IFNγ for time periods ranging from 4-8 hours. Cell lysates were immunoprecipitated with anti-$p21^{WAF1/CIP1}$ antibodies and the presence of Cdk2 was determined by immunoblotting (Figure 3-8). In T98G cultures, $p21^{WAF1/CIP1}$-associated Cdk2 levels were higher in IFNγ-treated cells than in untreated cells at 8 hours, which is consistent with the growth inhibition by IFNγ

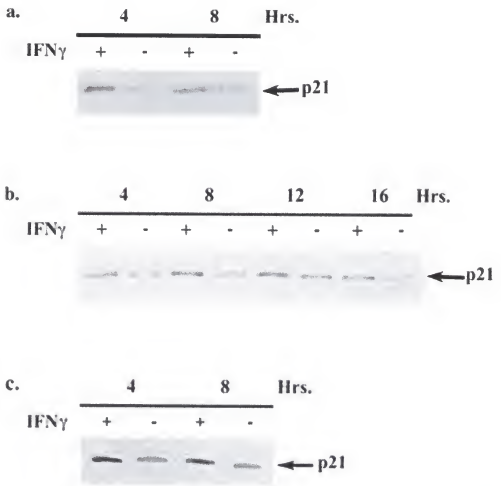


Figure 3-7: IFNγ Treatment of Asynchronous Cells Increases $p21^{WAF1/CIP1}$ Production in (a) T98G, (b) SNB-19, and (c) U-373 cells. Cells were grown for 24 hours in complete media and subsequently incubated from 4 - 16 hours with or without 2500 units/ml IFNγ. Immunoblot analysis was performed on cell lysates using anti-$p21^{WAF1/CIP1}$ antibodies as described in Materials and Methods. $p21^{WAF1/CIP1}$ levels in T98G and U-373 IFNγ-treated cells dropped to those seen in untreated cells at both 12 and 16 hours (data not shown).

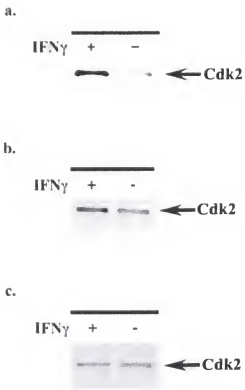


Figure 3-8: IFNγ Treatment Leads to Increased Binding of $p21^{WAF1/CIP1}$ to Cdk2 in (a) T98G, (b) SNB-19, and (c) U-373 Cells. Cells were grown for 24 hours in starvation media and subsequently incubated over a time period ranging from 4 - 8 hours with or without 2500 units/ml IFNγ. Cell lysates were immunoprecipitated using anti-$p21^{WAF1/CIP1}$ antibodies and immunoblotted using anti-Cdk2 antibodies as described in Material and Methods. Results seen at 4 hours (data not shown) were similar to those seen at 8 hours (shown above).

seen in these cultures. IFNγ-treated SNB-19 cells showed significant levels of $p21^{WAF1/CIP1}$-Cdk2 complex as compared to untreated cells, although, these levels were lower than those found in T98G cells. The $p21^{WAF1/CIP1}$-associated Cdk2 levels in IFNγ-treated U-373 cells were relatively low compared to those seen in T98G and SNB-19 cells. Thus, the amount of $p21^{WAF1/CIP1}$ complexed to Cdk2-cyclin for the cell lines correlated with the levels produced in response to IFNγ treatment.

Inhibition of Cdk2 Activity

We next performed kinase assays to determine whether the increased binding of $p21^{WAF1/CIP1}$ to Cdk2 correlated with reduced kinase activity. Cell lysates from glioblastoma cells treated with IFNγ were immunoprecipitated with anti-Cdk2 antibodies and Cdk2 activity was assessed (Tables 3-2 - 3-4). The data are displayed alongside cell cycle data from Figures 3-3 – 3-5 for ease of interpretation. Cells treated with IFNγ showed reduced kinase activity compared to untreated cells, correlating with inhibition of cell proliferation and increases in $p21^{WAF1/CIP1}$. T98G cells showed the greatest reduction in Cdk2 activity in response to IFNγ, consistent with the fact that T98G cells were the most inhibited by IFNγ and showed the strongest cell cycle delay following IFNγ treatment. SNB-19 cells showed a reduction in Cdk2 activity that increased at later time points. This is consistent with the pronounced S phase inhibition of the cell cycle. Cdk2 activity in U-373 cells was reduced following IFNγ treatment, but to a lesser extent than was seen in the other two cell lines. This correlates with the moderate antiproliferative effects and cell cycle delay seen in this cell line following IFNγ treatment. Thus, these results show that $p21^{WAF1/CIP1}$ produced in response to IFNγ treatment binds Cdk2 and

Table 3-2: Effects of IFNγ on Cdk2 Activity in T98G[a]

Time (hrs)	IFNγ (U/ml)	(%) of cells in			Cdk2 Activity	
		G_o/G_1	S	G_2/M	CPM	% Reduction
4	0	88.0	4.9	7.1	243982	-
	2500	88.3	1.7	10.1	134164	45.0
8	0	86.7	5.7	7.7	1027767	-
	2500	89.6	4.6	5.8	155169	84.9
12	0	74.1	23.3	2.6	458670	-
	2500	84.6	5.8	9.6	284997	37.9
16	0	48.4	44.5	7.2	600056	-
	2500	77.9	13.1	9.0	215534	64.1
20	0	38.3	52.4	9.4		
	2500	53.0	39.9	7.0		
24	0	49.7	17.1	33.2	885220	-
	2500	48.9	36.7	14.4	906930	-

[a]T98G cells were treated with or without 2500 units/ml IFNγ for 4, 8, 12, 16, and 24 hrs. Cell lysates were immunoprecipitated with anti-Cdk2 antibody and Cdk2 activity was assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as c.p.m. with corresponding percent of control. Basal c.p.m. was 475280. Cell cycle data from figure 3-2 is included for ease of interpretation. The doubling time of T98G is approximately 25 hours, as observed in cell culture.

Table 3-3: Effects of IFNγ on Cdk2 Activity in SNB-19[a]

Time (hrs)	IFNγ (U/ml)	(%) of cells in			Cdk2 Activity	
		G_o/G_1	S	G_2/M	CPM	% Reduction
10	0	70.1	16.1	13.8	157385	-
	2500	73.3	13.1	13.6	96518	36.9
20	0	40.8	47.5	11.6	617912	-
	2500	52.3	38.9	8.8	142215	77.0
24	0	39.1	44.1	16.8	295274	-
	2500	39.3	50.2	10.5	117410	60.2
28	0	46.2	31.3	22.5	199723	-
	2500	42.3	47.9	9.8	205035	-
32	0	60.3	24.8	14.9	159989	-
	2500	37.9	51.5	10.6	217810	-

[a]SNB-19 cells were treated with or without 2500 units/ml IFNγ for 10, 20, 24, 28, and 32 hrs. Cell lysates were immunoprecipitated with anti-Cdk2 antibody and Cdk2 activity was assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as c.p.m. with corresponding percent of control. Basal c.p.m. was 35357. Cell cycle data from figure 3-4 is included for ease of interpretation. The doubling time of SNB-19 is approximately 36 hours, as observed in cell culture.

Table 3-4: Effects of IFNγ on Cdk2 Activity in U-373[a]

Time (hrs)	IFNγ (U/ml)	(%) of cells in			Cdk2 Activity	
		G_0/G_1	S	G_2/M	CPM	% Reduction
12	0	72.1	22.0	5.9	171901	-
	2500	79.2	11.5	9.4	96955	40.6
16	0	36.9	45.9	17.2	342345	-
	2500	42.9	47.1	10.0	171411	50.0
20	0	29.9	50.3	19.8	638140	-
	2500	31.8	49.5	18.7	309021	51.6
24	0	38.7	37.8	23.5	890200	-
	2500	28.3	54.1	17.6	444089	50.1
28	0	50.8	26.6	22.7	450203	-
	2500	40.0	30.0	30.0	324942	27.8

[a]U-373 cells were treated with or without 2500 units/ml IFNγ for 12, 16, 20, 24, and 28 hrs. Cell lysates were immunoprecipitated with anti-Cdk2 antibody and Cdk2 activity was assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as c.p.m. with corresponding percent reduction. Basal c.p.m. was 61915. Cell cycle data from figure 3-3 is included for ease of interpretation. The doubling time of U-373 is approximately 26 hours, as observed in cell culture.

reduces Cdk2 activity proportionally to inhibition of cell growth. Taken together, these results show that IFNγ inhibition of the glioblastoma cell cycle correlates both in timing and magnitude with increased levels of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, strongly suggesting that $p21^{WAF1/CIP1}$ plays a role in mediating these antiproliferative effects.

Combined Inhibitory Effect of IFNγ and Acyclovir on the Glioblastoma Cell Cycle

IFNγ Inhibition of Cell Growth

We began our studies into the combined inhibitory effects of IFNγ and the nucleoside analog acyclovir by determining their effects, both alone and in combination, on the glioblastoma cell lines T98G, SNB-19, and U-373. As previously indicated, glioblastoma multiforme is derived from astrocytes. Astrocytes are extremely important as they provide both physical and nutritional support to neurons of the central nervous system. Thus, we also wanted to determine the effects of IFNγ and ACV on normal human astrocytes. Cells were treated with or without IFNγ at concentrations of 625, 1250, 2500, 5000 and 10000 units/ml over a period of 96 hours. Cells were subsequently harvested and inhibition of cell growth was determined by direct cell count. As seen in Figure 3-9, IFNγ possesses strong antiproliferative activities on the glioblastoma cell lines T98G, SNB-19, and U-373. On the other hand, IFNγ did not affect the growth of normal human astrocytes at concentrations up to 5000 units/ml. At 10000 units/ml astrocyte cell growth was inhibited by 25.4% as compared to 54.3-82.5% for the glioblastoma cell lines tested. The cell viability at all concentrations was approximately 92%, suggesting that

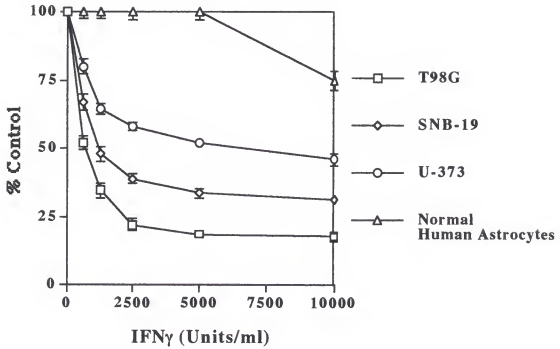


Figure 3-9: Antiproliferative Effect of IFNγ on Normal Human Astrocytes. Cells (1.0×10^5 cells/well) were incubated with or without IFNγ at concentrations of 625, 1250, 2500, 5000, and 10000 units/ml for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. Cell numbers at 100% control for T98G, SNB-19, U-373, and normal human astrocytes were 7.4, 6.3, 6.45, and 3.15×10^5 cells, respectively. The percent viability at all concentrations was approximately 92%. Data from three representative experiments are expressed as total number of cells per sample +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of 10000 units/ml IFNγ for 96 hours ($P<0.04$).

inhibition of cell growth was not due to cell death. In our previous studies we have shown that IFNγ treatment of glioblastoma cell lines at concentrations from 625 to 10000 units/ml resulted in significant inhibition of cell growth with percent inhibition values averaging from 34.3 to 63.4%, respectively. Thus, the glioblastoma cell lines appear much more sensitive to IFNγ than normal human astrocytes.

Acyclovir Inhibition of Cell Growth

We next determined the antiproliferative effect of ACV on the glioblastoma cell lines T98G, SNB-19, and U-373 as well as on normal human astrocytes. Cells were treated with or without ACV at concentrations of 100, 250, and 500 μg/ml over a time period of 96 hours and growth inhibition was determined by direct cell count. We found that the growth of all cell lines was inhibited in a dose-dependent manner (Figure 3-10). T98G, SNB-19, and U-373 cell growth was maximally inhibited at 500 μg/ml, with percent inhibition values of 66.8, 57.3, and 68.3%, respectively (Table 3-5). Astrocyte cell growth was once again less inhibited than glioblastoma cell growth, with percent inhibition values of 21.0, 30.9, and 38.3%, respectively (Table 3-5). The viability for all cell lines was approximately 90%.

Phosphorylation of Acyclovir by Cellular Kinases

Nucleoside analogs have been popular in the treatment of several viral diseases due to their "specificity" against virally infected cells. Nucleoside analogs must be converted to their triphosphate form to become active in the cell. It has been suggested that ACV, an analog of guanosine, can only be phosphorylated by

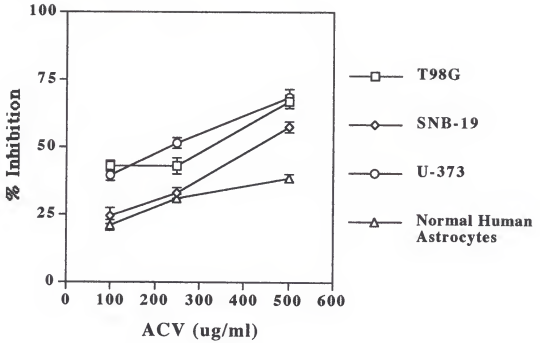


Figure 3-10: Antiproliferative Effect of ACV on Normal Human Astrocytes. Cells (1.0×10^5 cells/well) were incubated with or without ACV at concentrations of 100, 250, and 500 ug/ml for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability at all concentrations was approximately 93%. Data from three representative experiments are expressed as total number of cells per sample +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of 100, 250, and 500 ug/ml ACV for 96 hours in T98G ($P<0.03$), SNB-19 ($P<0.02$), U-373 ($P<0.03$), and normal human astrocytes ($P<0.02$).

Table 3-5: Inhibition of Glioblastoma Cell Line Proliferation[a]

		% Inhibition			
Time (hrs)	ACV(ug/ml)	T98G	SNB-19	U-373	NHA
96	100	42.7	24.1	39.2	21.0
	250	42.7	32.9	51.2	30.9
	500	66.8	57.3	68.3	38.3

[a]Data are from three representative experiments performed in triplicate. Percent inhibition values were calculated directly from cell count experiments using 100, 250, and 500 ug/ml ACV.

the viral thymidine kinase (TK). However, it has been shown that guanosine can also be phosphorylated by cellular deoxyguanosine and deoxycytidine kinases albeit with less efficiency than TK (Kawasaki et al., 1992; Sarup and Fridland, 1987; Sjoberg et al., 1998). In fact, previous studies have suggested that TKs of herpesviruses may have evolved from cellular deoxycytidine kinases (Harrison et al., 1991). To be sure that ACV was being phosphorylated in the glioblastoma cell lines T98G, SNB-19, and U-373, we performed an assay for kinase activity. Samples containing cell lysate were incubated with [8-^{3}H]-ACV and subsequently filtered through DE-81 filter disks. Phosphorylation was determined by quantifying the radioactivity remaining on filter disks using a scintillation counter. We found that the glioblastoma cell lines were able to phosphorylate ACV as compared to controls containing no cell lysate (Figure 3-11). Phosphorylation appeared to be specific as competition with cold ACV resulted in counts similar to controls (data not shown). The ability of normal human astrocytes to phosphorylate ACV was 7-fold lower than that of the glioblastoma cell lines. This correlates with the reduced effect of ACV on normal human astrocytes as compared to the glioblastoma cell lines.

Cell Cycle Analysis Following Acyclovir Treatment

We next determined the effect of ACV on the glioblastoma cell cycle of T98G cells. As determined by cell count data, 500 μg/ml appeared to be the lowest concentration of ACV that achieved maximal inhibition and was used in subsequent cell cycle experiments. T98G cells were synchronized in the G_0/G_1 phase of the cell cycle by serum starvation and subsequently treated with or without 500 μg/ml of ACV over a time period of 12 to 48 hours. We found that the progress of cells

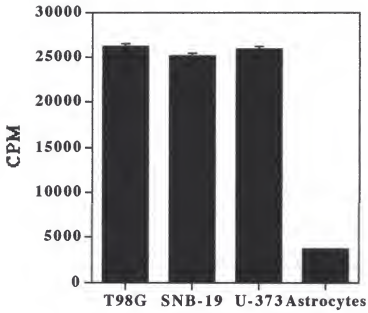


Figure 3-11: Phosphorylation of [8-^{3}H]-ACV by T98G, SNB-19, U-373, and Normal Human Astrocytes. Samples containing cell lysate were incubated with [8-^{3}H]-ACV and subsequently filtered through DE-81 filter disks. Filter disks were washed and phosphorylation was determined by reading radioactivity remaining on filter disks using a scintillation counter.

treated with ACV through the cell cycle was strongly delayed as determined by flow cytometry analysis (Table 3-6). The delay began at the G_1/S transition of the cell cycle and continued throughout the S phase. As ACV acts as a mandatory DNA chain terminator one would expect to see a delay in the S phase of the cell cycle. Similar results were obtained with SNB-19 and U-373 cells (Tables 3-7 and 3-8). Thus, the inhibitory effects of ACV correlate with a delay/block in the S phase.

Combined Effect of IFNγ and Acyclovir on Cell Growth

In previous studies we have shown that the antiproliferative effects of IFNγ on the glioblastoma cell lines T98G, SNB-19, and U-373 are accompanied by strong cell cycle delays occurring at the G_1 to S phases. Considering that the cell cycle delay induced by ACV treatment partially overlaps that seen following IFNγ treatment, we wanted to determine the combined effects of IFNγ and ACV on these cell lines. Cells were treated with various suboptimal concentrations of IFNγ and ACV, both alone and in combination, over a period of 96 hours and inhibition of cell growth was determined by direct cell count. All combinations of IFNγ and ACV inhibited the growth of all cell lines in an additive or slightly greater than additive manner with inhibition values reaching as high as 60.3, 55.0, and 40.5% for the cell lines T98G, SNB-19, and U-373, respectively (Figures 3-12 – 3-14). Of the various suboptimal concentrations used, 30 units/ml IFNγ and 25 μg/ml ACV were found to best illustrate the combined antiproliferative effect and were therefore used in subsequent experiments. This shows that suboptimal concentrations of IFNγ combined with ACV were able to achieve a similar antiproliferative effect as that seen with optimal concentrations of either agent alone. This finding may be

Table 3-6: Cell Cycle Analysis of Acyclovir-Treated T98G Cells[a]

Time (hours)	ACV (ug/ml)	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	78.6	14.4	7.0
	500	85.1	11.0	3.9
16	---	65.6	34.3	0.1
	500	86.2	11.0	2.8
20	---	49.6	50.3	0.1
	500	81.8	18.2	0.0
24	---	58.8	17.6	23.6
	500	71.4	25.8	2.8
36	---	90.9	5.6	3.5
	500	19.8	80.2	0.0
48	---	77.6	17.2	5.2
	500	20.0	77.2	2.8

[a]ACV inhibits the progression of T98G cells through the cell cycle causing a strong delay at the G_1/S transition continuing throughout the S phase. T98G cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

Table 3-7: Cell Cycle Analysis of Acyclovir-Treated SNB-19 Cells[a]

Time (hours)	ACV (ug/ml)	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	71.5	22.7	5.8
	500	81.6	13.2	5.2
16	---	52.9	41.4	5.7
	500	78.5	17.4	4.1
20	---	38.8	43.5	17.7
	500	70.8	29.2	0.0
24	---	44.6	30.7	24.7
	500	37.1	61.6	1.3
36	---	69.5	19.4	11.1
	500	21.5	57.5	21.0
48	---	73.6	18.2	8.2
	500	50.5	30.2	19.3

[a]ACV inhibits the progression of SNB-19 cells through the cell cycle causing a strong delay at the G_1/S transition continuing throughout the S phase. SNB-19 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

Table 3-8: Cell Cycle Analysis of Acyclovir-Treated U-373 Cells[a]

Time (hours)	ACV (ug/ml)	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	81.9	14.8	3.3
	500	85.7	11.9	2.4
16	---	54.9	45.1	0.0
	500	89.3	9.8	0.9
20	---	36.0	46.7	17.4
	500	84.1	15.7	0.2
24	---	69.4	9.6	21.0
	500	73.7	26.3	0.0
36	---	70.7	21.7	7.6
	500	32.3	38.2	29.5
48	---	73.8	19.5	6.7
	500	50.9	29.1	20.0

[a]ACV inhibits the progression of U-373 cells through the cell cycle causing a strong delay at the G_1/S transition continuing throughout the S phase. U-373 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

Figure 3-12: IFNγ and ACV Inhibit Cellular Proliferation of T98G Cells In an Approximately Additive Manner. Cells (1.0×10^5 cells/well) were incubated with or without 30 units/ml IFNγ and 25 μg/ml ACV (as shown) for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability was approximately 90%. Data from three representative experiments are expressed as % inhibition +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of indicated amounts of IFNγ and ACV for 96 hours ($P<0.03$).

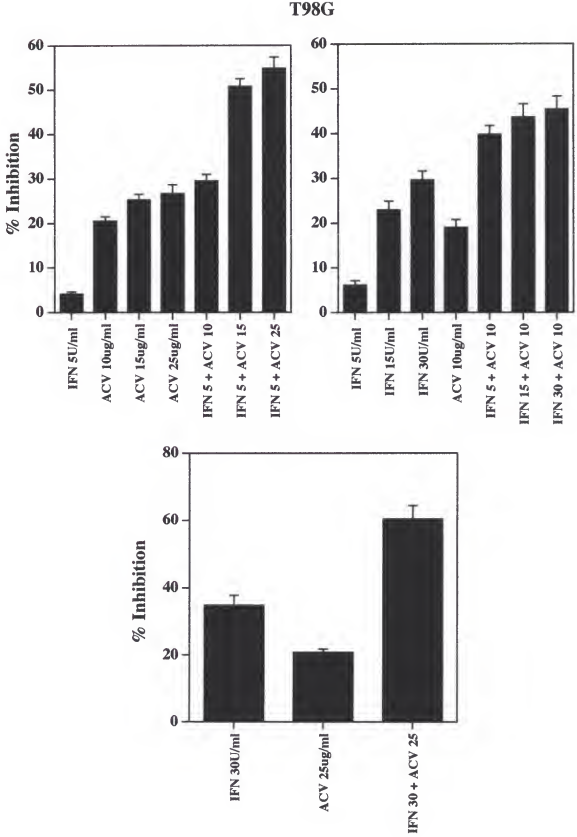

T98G
% Inhibition
0
10
20
30
40
50
60
IFN 5U/ml
ACV 10ug/ml
ACV 15ug/ml
ACV 25ug/ml
IFN 5 + ACV 10
IFN 5 + ACV 15
IFN 5 + ACV 25
IFN 5U/ml
IFN 15U/ml
IFN 30U/ml
ACV 10ug/ml
IFN 5 + ACV 10
IFN 15 + ACV 10
IFN 30 + ACV 10
% Inhibition
0
20
40
60
80
IFN 30U/ml
ACV 25ug/ml
IFN 30 + ACV 25

Figure 3-13: IFNγ and ACV Inhibit Cellular Proliferation of SNB-19 Cells In an Approximately Additive Manner. Cells (1.0 x 10^5 cells/well) were incubated with or without 30 units/ml IFNγ and 25 μg/ml ACV (as shown) for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability was approximately 92%. Data from three representative experiments are expressed as % inhibition +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of indicated amounts of IFNγ and ACV for 96 hours ($P<0.04$).

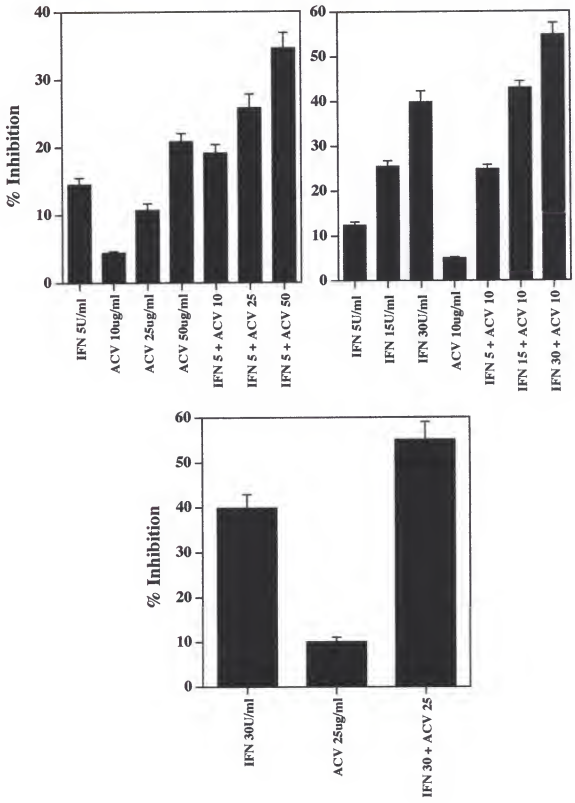
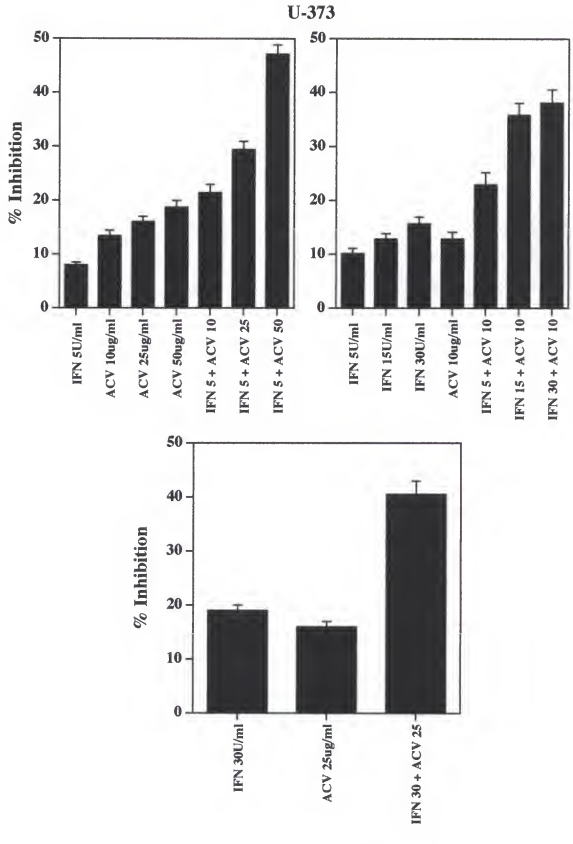

SNB-19
% Inhibition
0
10
20
30
40
IFN 5U/ml
ACV 10ug/ml
ACV 25ug/ml
ACV 50ug/ml
IFN 5 + ACV 10
IFN 5 + ACV 25
IFN 5 + ACV 50
0
10
20
30
40
50
60
IFN 5U/ml
IFN 15U/ml
IFN 30U/ml
ACV 10ug/ml
IFN 5 + ACV 10
IFN 15 + ACV 10
IFN 30 + ACV 10
% Inhibition
0
10
20
30
40
50
60
IFN 30U/ml
ACV 25ug/ml
IFN 30 + ACV 25

Figure 3-14: IFNγ and ACV Inhibit Cellular Proliferation of U-373 Cells In an Approximately Additive Manner. Cells (1.0×10^5 cells/well) were incubated with or without 30 units/ml IFNγ and 25 μg/ml ACV (as shown) for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability was approximately 91%. Data from three representative experiments are expressed as % inhibition +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of indicated amounts of IFNγ and ACV for 96 hours ($P<0.03$).


U-373
% Inhibition
50
40
30
20
10
0
IFN 5U/ml
ACV 10ug/ml
ACV 25ug/ml
ACV 50ug/ml
IFN 5 + ACV 10
IFN 5 + ACV 25
IFN 5 + ACV 50
IFN 5U/ml
IFN 15U/ml
IFN 30U/ml
ACV 10ug/ml
IFN 5 + ACV 10
IFN 15 + ACV 10
IFN 30 + ACV 10
% Inhibition
IFN 30U/ml
ACV 25ug/ml
IFN 30 + ACV 25

significant considering the potential toxicity of IFNγ and ACV at high concentrations. Normal human astrocyte cell growth did not appear to be inhibited by any concentration of IFNγ used, but was inhibited by ACV at concentrations of 25 μg/ml and 50 μg/ml, with inhibition values of 11.1 and 17.3%, respectively (Figure 3-15). The combination of IFNγ and ACV resulted in growth inhibition that was approximately the same as that seen in cells treated with ACV alone. Therefore the combined antiproliferative effects observed appeared to be due solely to the effects of ACV. Consistent with the effects of IFNγ and ACV alone, the combined effect of IFNγ and ACV on glioblastoma cell lines was approximately 3-fold greater than that on normal human astrocytes.

Cell Cycle Analysis Following IFNγ and Acyclovir Treatment

The combined effects of IFNγ and ACV were further analyzed by flow cytometry. T98G cells were synchronized by serum starvation and subsequently treated with a combination of IFNγ (30 units/ml) and ACV (25 μg/ml) over a period of 12 to 48 hours. Cell cycle analysis showed that treatment with the combination of IFNγ and ACV led to a stronger delay in the cell cycle of all cell lines than that seen with either IFNγ or ACV alone (Table 3-9). This is consistent with the combined antiproliferative effect of IFNγ and ACV. Further, the cell cycle delay following combined treatment with IFNγ and ACV appears to begin early in the G_1 phase and continue throughout the S phase. This correlates with the individual effects of each agent as IFNγ primarily effects the G_1 phase while ACV predominantly affects the S phase of the cell cycle. Similar results were obtained with IFNγ/ACV-treated SNB-19 and U-373 cells (Tables 3-10 and 3-11). Thus, IFNγ and ACV appear to be

Figure 3-15: $IFN\gamma$ and ACV Inhibit Cellular Proliferation of Normal Human Astrocytes In an Approximately Additive Manner. Cells (1.0×10^5 cells/well) were incubated with or without 30 units/ml $IFN\gamma$ and 25 μg/ml ACV (as shown) for 96 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. The percent viability was approximately 90%. Data from three representative experiments are expressed as % inhibition +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of indicated amounts of $IFN\gamma$ and ACV for 96 hours ($P<0.04$).

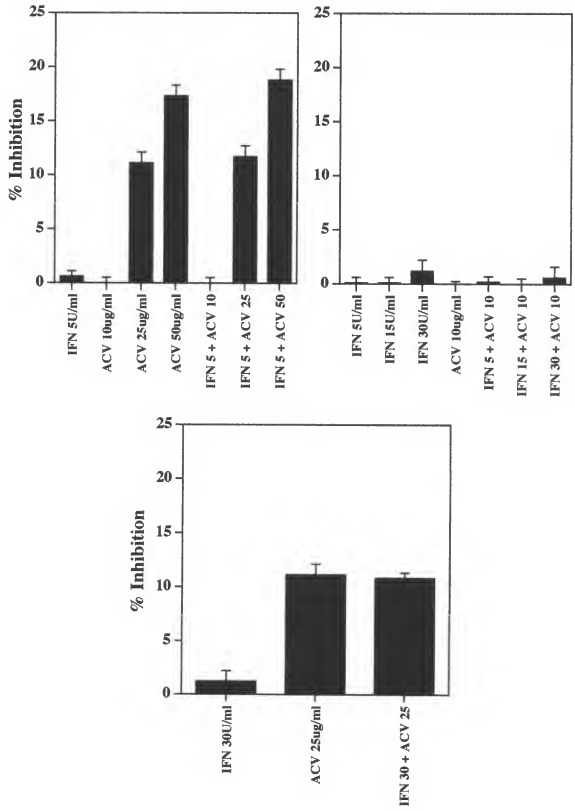

Normal Human Astrocytes
% Inhibition
25
20
15
10
5
0
IFN 5U/ml
ACV 10ug/ml
ACV 25ug/ml
ACV 50ug/ml
IFN 5 + ACV 10
IFN 5 + ACV 25
IFN 5 + ACV 50
IFN 5U/ml
IFN 15U/ml
IFN 30U/ml
ACV 10ug/ml
IFN 5 + ACV 10
IFN 15 + ACV 10
IFN 30 + ACV 10
% Inhibition
IFN 30U/ml
ACV 25ug/ml
IFN 30 + ACV 25

Table 3-9: Cell Cycle Analysis of IFNγ and Acyclovir-Treated T98G Cells[a]

Time (hours)	Treatment	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	85.2	12.3	2.5
	ACV 25ug/ml	87.5	10.7	1.8
	IFNγ 30U/ml	89.4	5.5	5.1
	IFNγ + ACV	88.4	8.6	3.0
16	---	61.0	37.9	1.1
	ACV 25ug/ml	61.2	38.8	0.0
	IFNγ 30U/ml	77.8	22.2	0.0
	IFNγ + ACV	80.9	15.3	3.8
20	---	49.3	43.2	7.5
	ACV 25ug/ml	44.6	55.0	0.4
	IFNγ 30U/ml	50.1	49.7	0.2
	IFNγ + ACV	59.3	40.7	0.0
24	---	46.4	32.7	20.9
	ACV 25ug/ml	35.9	50.2	13.9
	IFNγ 30U/ml	29.2	53.2	17.6
	IFNγ + ACV	31.8	68.2	0.0
36	---	87.1	10.1	2.8
	ACV 25ug/ml	82.2	13.4	4.4
	IFNγ 30U/ml	83.2	13.2	3.6
	IFNγ + ACV	67.4	21.8	10.8
48	---	70.6	21.9	7.5
	ACV 25ug/ml	57.6	34.0	8.4
	IFNγ 30U/ml	64.7	23.8	11.5
	IFNγ + ACV	54.6	38.9	6.5

[a]IFNγ combined with ACV inhibits the progression of T98G cells through the cell cycle causing a strong delay in the G_1 and S phases. T98G cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

Table 3-10: Cell Cycle Analysis of IFNγ and Acyclovir-Treated SNB-19 Cells[a]

Time (hours)	Treatment	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	75.7	19.4	4.9
	ACV 25ug/ml	77.1	17.1	5.8
	IFNγ 30U/ml	78.9	15.4	5.7
	IFNγ + ACV	79.2	16.3	4.5
16	---	39.3	57.7	3.0
	ACV 25ug/ml	49.6	50.4	0.0
	IFNγ 30U/ml	57.5	40.0	2.5
	IFNγ + ACV	58.7	40.6	0.7
20	---	23.8	58.7	17.5
	ACV 25ug/ml	18.2	76.8	5.0
	IFNγ 30U/ml	27.3	65.2	7.5
	IFNγ + ACV	32.0	67.8	0.2
24	---	35.7	26.0	38.3
	ACV 25ug/ml	24.3	37.6	38.1
	IFNγ 30U/ml	27.4	42.9	29.7
	IFNγ + ACV	23.1	63.6	13.3
36	---	68.0	23.8	8.2
	ACV 25ug/ml	66.0	26.6	7.4
	IFNγ 30U/ml	69.1	16.9	14.0
	IFNγ + ACV	52.2	26.3	21.5
48	---	73.0	17.3	9.7
	ACV 25ug/ml	66.0	24.1	9.9
	IFNγ 30U/ml	82.3	7.5	10.2
	IFNγ + ACV	68.8	16.0	15.2

[a]IFNγ combined with ACV inhibits the progression of SNB-19 cells through the cell cycle causing a strong delay in the G_1 and S phases. SNB-19 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

Table 3-11: Cell Cycle Analysis of IFNγ and Acyclovir-Treated U-373 Cells[a]

Time (hours)	Treatment	Cell Cycle Phase (%) G_0/G_1	S	G_2/M
12	---	82.5	11.1	6.4
	ACV 25ug/ml	75.7	16.3	8.0
	IFNγ 30U/ml	88.1	4.1	7.8
	IFNγ + ACV	80.4	10.8	8.8
16	---	63.2	35.8	1.0
	ACV 25ug/ml	70.8	24.9	4.3
	IFNγ 30U/ml	89.9	7.5	2.6
	IFNγ + ACV	85.8	7.4	6.8
20	---	27.6	71.0	1.4
	ACV 25ug/ml	31.6	68.4	0.0
	IFNγ 30U/ml	59.8	40.2	0.0
	IFNγ + ACV	69.1	30.9	0.0
24	---	30.4	46.3	23.3
	ACV 25ug/ml	24.3	56.2	19.5
	IFNγ 30U/ml	13.9	77.7	8.4
	IFNγ + ACV	19.0	81.0	0.0
36	---	50.4	38.4	11.2
	ACV 25ug/ml	49.6	43.5	6.9
	IFNγ 30U/ml	54.8	41.9	3.3
	IFNγ + ACV	67.1	24.6	8.3
48	---	58.6	35.0	6.4
	ACV 25ug/ml	56.5	36.4	7.1
	IFNγ 30U/ml	54.1	38.3	7.6
	IFNγ + ACV	42.7	53.3	4.0

[a]IFNγ combined with ACV inhibits the progression of U-373 cells through the cell cycle causing a strong delay in the G_1 and S phases. U-373 cells were synchronized in G_0/G_1 by growing cells in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide staining followed by flow cytometry analysis.

working together at different sites in the cell cycle to further slow the cell cycle compared to their effects alone.

IFNγ Induction of $p21^{WAF1/CIP1}$ Leads to Increased Binding of $p21^{WAF1/CIP1}$ to PCNA

In previous studies we have shown that the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ may play a role in the antiproliferative effects of IFNγ seen in the G_1 to S phases of the cell cycle in the glioblastoma cell lines T98G, SNB-19, and U-373 through its interactions with Cdk2. Interestingly, multiple $p21^{WAF1/CIP1}$ molecules bound to PCNA can prevent it from activating DNA polymerase δ, thus inhibiting DNA synthesis. We therefore determined if IFNγ treatment was able to increase the binding of $p21^{WAF1/CIP1}$ to PCNA in the glioblastoma cell lines T98G, SNB-19, and U-373. Cells were treated with or without IFNγ (30 units/ml) over a time period ranging from 4 to 16 hours. Cell lysates were immunoprecipitated with anti-$p21^{WAF1/CIP1}$ antibodies and the presence of PCNA was determined by immunoblotting (Figure 3-16). In T98G cells, IFNγ-treated samples showed increased binding of $p21^{WAF1/CIP1}$ to PCNA at the 4 and 8-hour time points as compared to untreated samples. This pattern was also seen in U-373 cells, although levels of $p21^{WAF1/CIP1}$ bound to PCNA were lower than that seen in T98G cells. SNB-19 cells showed an increase in $p21^{WAF1/CIP1}$ bound to PCNA in IFNγ-treated samples as well and this was seen from 4 to 16 hours. Although the levels and timing of $p21^{WAF1/CIP1}$ binding to PCNA differed slightly between cell lines, these levels correlated with both the amount of $p21^{WAF1/CIP1}$ produced as well as the cell cycle delays seen following IFNγ treatment in each cell line. Correspondingly, this

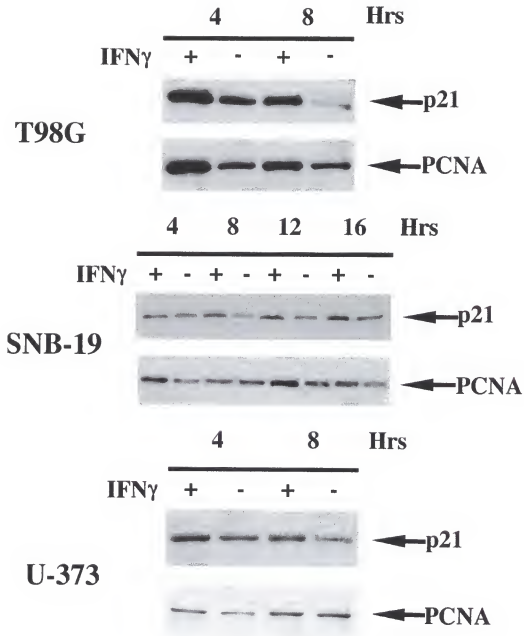


Figure 3-16: IFNγ Treatment Leads to Increased Binding of $p21^{WAF1/CIP1}$ to PCNA in T98G, SNB-19, and U-373 Cells. Cells were grown for 24 hours in serum-free media and subsequently incubated over a time period ranging from 4 to 16 hours with or without 30 units/ml IFNγ. Cell lysates were immunoprecipitated using anti-$p21^{WAF1/CIP1}$ antibodies and immunoblotted using anti-PCNA antibodies as described in Materials and Methods.

increase in $p21^{WAF1/CIP1}$ binding to PCNA may contribute to the delay seen in the S phase of the cell cycle. Therefore, we have shown another possible mechanism by which IFNγ may exert its antiproliferative effects, which may also play a role in the combined effect of IFNγ and ACV as both inhibit the cell cycle through different mechanisms. Most importantly, the combination IFNγ and ACV is able to significantly inhibit glioblastoma cell growth in an additive manner while not significantly inhibiting the growth of normal human astrocytes.

IFNγ Inhibition of Prostate Cancer Cell Proliferation

Inhibition of Cell Growth

We began our studies on prostate cancer by determining the antiproliferative effects of IFNγ on the DU145, LNCaP, and PC-3 prostate cancer cell lines. Cells were grown in the presence or absence of 5000 units/ml IFNγ over a period of 72 hours and the total number of cells was determined by direct cell count. IFNγ inhibited DU145 and PC-3 cell growth by up to 59.1% and 50.8%, respectively, while no inhibition of LNCaP cells was seen (Table 3-12). We also found that IFNγ inhibited DU145 and PC-3 cells in a dose-dependent manner (Figure 3-17). DU145 and PC-3 cells were treated in the presence of 0, 500, 1000, and 5000 units/ml IFNγ for 72 hours and the total number of cells was determined. Thus, the proliferation of the DU145 and PC-3 cell lines was inhibited by IFNγ while LNCaP cells did not appear to be affected.

Table 3-12: Inhibition of Prostate Cancer Cell Growth by IFN_γ

Cell Line	IFN_γ (U/ml)	Total # Cells (x 10^5)	Inhibition (%)
DU145	0	13.2 ± .41	--
	5000	5.40 ± .11	59.1 ± 2.1
LNCaP	0	1.50 ± .11	--
	5000	1.80 ± .18	--
PC-3	0	2.36 ± .21	--
	5000	1.16 ± .06	50.8 ± 1.9

[a]Cells were plated into 6 well plates (5 x 10^4 cells/well), grown in the presence or absence of IFN_γ for 72 hours and total number of cells determined. The percent viability for all cell lines was approximately 92%. Data are from three representative experiments performed in triplicate. Statistical significance was shown by Student's *t*-test for the difference in cell number grown in the presence and absence of IFN_γ ($p<0.05$).

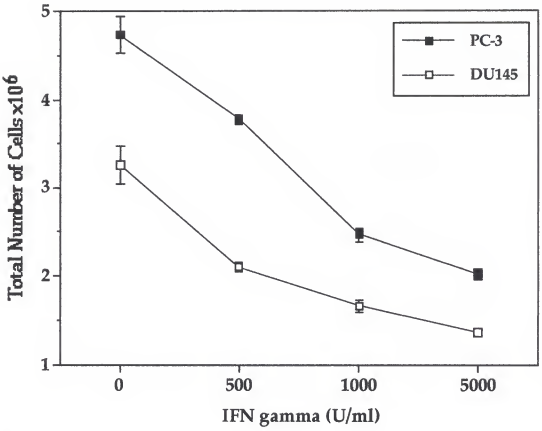


Figure 3-17: IFNγ Inhibits Cell Growth in DU145 and PC-3 Cells in a Dose-Dependent Manner. DU145 and PC-3 cells were treated with 500, 1000, 5000 units/ml IFNγ or media alone for 72 hours. Cells were then harvested, washed, and counted using trypan blue exclusion (0.4%) and total number of cells determined. Cell viability for all cell lines tested was approximately 92%. Data are from three representative experiments performed in triplicate. Statistical significance was shown by Student's t-test for the difference in cell number grown in the presence or absence of 500 units/ml ($p<0.05$), 1000 units/ml ($p<0.04$), and 5000 units/ml ($p<0.05$) IFNγ.

Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$

It has previously been shown that growth inhibition of the DU145 cells by IFNγ was due to the upregulation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Hobeika et al, 1998). $p21^{WAF1/CIP1}$ regulates the mammalian cell cycle by directly binding Cdks, which are necessary for progression of the cell cycle, and blocking their kinase activity. IFNγ upregulation of $p21^{WAF1/CIP1}$ describes a mechanism by which IFNγ inhibits the cell cycle and subsequent cell growth. DU145, LNCaP, and PC-3 cells were treated with 5000 units/ml IFNγ for 16 and 24 hours and subsequently lysed and immunoblotted. IFNγ induced $p21^{WAF1/CIP1}$ production in both DU145 and PC-3, but not LNCaP cells (Figure 3-18). Consistent with our previous findings, IFNγ upregulation of $p21^{WAF1/CIP1}$ correlated with the inhibition of cell proliferation.

IFNγ-Induced Phosphorylation of STAT1

We next determined if the signal transduction pathway of IFNγ was intact in the DU145, LNCaP, and PC-3 cell lines. IFNγ initiates its cellular effects by binding to specific cell-surface receptors. Like other cytokines, this ligand-receptor interaction induces the phosphorylation of a group of proteins known as STATs, which in the absence of ligand, reside unphosphorylated in the cytoplasm (Darnell et al., 1994). IFNγ-mediated signal transduction specifically phosphorylates STAT1, which then acts directly to turn on IFNγ inducible genes. DU145, LNCaP, and PC-3 cells were treated with 5000 units/ml IFNγ for 10 minutes. Cell lysates were immunoprecipitated using antibodies specific for STAT1 and precipitates were subsequently immunoblotted with antibodies for both STAT1 and phosphorylated

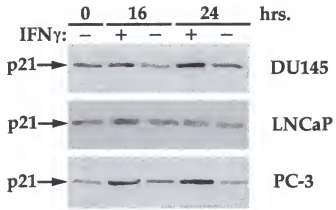


Figure 3-18: IFNγ Upregulates $p21^{WAF1/CIP1}$ Expression in DU145 and PC-3 But Not LNCaP Cells. DU145, LNCaP, and PC-3 cells were treated with 5000 units/ml IFNγ or media alone for 0, 16, and 24 hours. Cell lysates were then immunoblotted with antibodies specific for $p21^{WAF1/CIP1}$. Densitometric analysis of radiographic film showed a 1.5 and 1.2-fold increase in $p21^{WAF1/CIP1}$ expression for DU145 cells and a 1.2 and 2.5-fold increase for PC-3 cells at 16 and 24 hours, respectively, in the presence of IFNγ. No increase in $p21^{WAF1/CIP1}$ expression was seen in LNCaP cells.

STAT1. Both DU145 and PC-3 cells had strong phosphorylation of STAT1 following IFNγ treatment while no STAT1 phosphorylation was seen in LNCaP cells (Figure 3-19). These results support the lack of response by the LNCaP cell line to IFNγ, since the JAK/STAT transduction pathway is not triggered in these cells. However, the transduction pathway is active in both DU145 and PC-3 cells, consistent with up-regulation of $p21^{WAF1/CIP1}$ and associated anti-proliferative effects seen in these cells.

Downregulation of neu/HER-2

Many of the mechanisms underlying the antiproliferative and cytotoxic effects of IFNγ on tumor cells remain to be elucidated. It has previously been shown that $p21^{WAF1/CIP1}$ plays a role in inhibiting the cell cycle of various transformed cell lines, but how IFNγ regulates proto-oncogenes is still unclear (Kominsky et al., 1998; Hobeika et al., 1998). The neu/HER-2 proto-oncogene has been implicated as a prognostic factor in several human malignancies including breast, ovarian, and prostate cancer. We looked at the effect of IFNγ on the downregulation of neu/HER-2 expression in the DU145, LNCaP, and PC-3 cell lines as a possible mechanism for inhibition of cell proliferation. Lysates from cells treated in the presence or absence of 5000 units/ml IFNγ for 96 hours were immunoblotted using antibodies specific for neu/HER-2. In DU145 and PC-3 cells, we saw a 61.6 and 78.7 percent reduction, respectively, in neu/HER-2 expression while no reduction in neu/HER-2 was seen in LNCaP cells (Figure 3-20). In addition, we saw a dose-dependent decrease in neu/HER-2 expression in DU145 and PC-3 cells (Figure 3-21). Cells were treated with 500, 1000, and 5000 units/ml IFNγ for 96 hours and cell lysates were

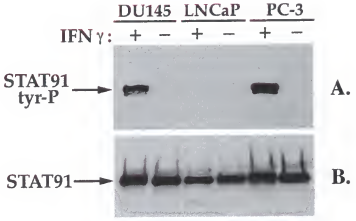


Figure 3-19: IFNγ Induces STAT1 Phosphorylation in DU145 and PC-3 But Not LNCaP Cells. Total cell lysates from DU145, PC-3, and LNCaP cells treated with or without 5000 units/ml IFNγ for 10 minutes were immunoprecipitated using anti-STAT1 antibodies. The immunoprecipitates were subjected to immunoblotting using antibodies for phosphorylated STAT1 (A). The immunoblot was then stripped of anti-phosphorylated STAT1 and reprobed using anti-STAT1 antibodies (B). The concentrations of STAT were similar for IFNγ-treated and untreated cells for all cell lines.

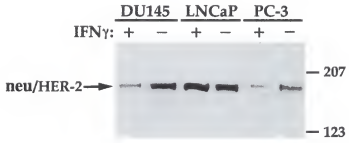


Figure 3-20: IFNγ Downregulates neu/HER-2 Expression in DU145 and PC-3 Cells. Cell lysates from DU145, PC-3 and LNCaP cells grown in the presence or absence of 5000 units/ml IFNγ for 4 days were immunoblotted with antibodies specific for neu/HER-2. Densitometric analysis of radiographic film showed a 61.6 and 78.7 percent decrease in neu/HER-2 expression for DU145 and PC-3 cells, respectively, while no difference was seen in LNCaP cells.

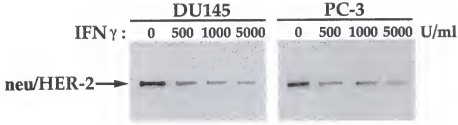


Figure 3-21: Downregulation of neu/HER-2 Expression by IFNγ in DU145 and PC-3 Cells is Dose-Dependent. Cell lysates from DU145 and PC-3 cells treated with 0, 500, 1000, or 5000 units/ml IFNγ for 4 days were immunoblotted with antibodies specific for neu/HER-2.

immunoblotted. Thus, IFNγ reduces neu/HER-2 expression in both the DU145 and PC-3 cells while no reduction is seen in the LNCaP cell line, consistent with the failure to phosphorylate STAT1 and induce $p21^{WAF1/CIP1}$ in the latter cell line.

Effect of p300 on IFNγ-Induced neu/HER-2 Downregulation

Although neu/HER-2 expression has been linked to tumor growth and metastasis, little is known about the transcriptional regulation of this oncogene. Previous findings have implicated the CREB-binding protein p300 as a transcriptional regulator of neu/HER-2 (Chen and Hung, 1997). The same studies have suggested that adenovirus E1A protein binding to p300 represses neu/HER-2 promoter activity. Interestingly, there have also been reports that STAT1 from IFNγ-treated cell lysates binds p300 at the E1A binding site (Zhang et al., 1996). We therefore wanted to determine whether p300 was involved in IFNγ-induced downregulation of neu/HER-2 expression. PC-3 cells were transfected with a plasmid containing the p300 gene (pRc/RSV-p300). Transfected and wildtype PC-3 cells were then grown in the presence or absence of 5000 units/ml IFNγ for 96 hours. Lysates were subsequently immunoblotted using antibodies specific for both p300 and neu/HER-2. Transfected cells showed a significant increase in p300 as compared to wildtype cells (Figure 3-22). Further, this increase in p300 production appeared to affect neu/HER-2 expression following IFNγ treatment as transfected cells showed only a 27.7% reduction in neu/HER-2 expression as compared to 78.7% in wildtype cells (Figure 3-23). This suggests that p300 may play a role in IFNγ-induced downregulation of neu/HER-2.

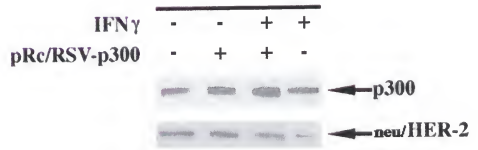


Figure 3-22: Transfection of PC-3 Cells With pRc/RSV-p300 Increases Expression of p300 and Decreases Downregulation of neu/HER-2 in Response to IFNγ Treatment. Cell lysates from PC-3 and PC-3 (pRc/RSV-p300) cells grown in the presence or absence of 5000 units/ml IFNγ for 4 days were immunoblotted with antibodies specific for p300 and neu/HER-2.

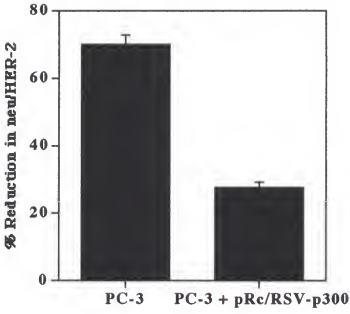


Figure 3-23: Densitometric Analysis of neu-HER-2 Expression. The percent reduction in neu/HER-2 expression following treatment with 5000 units/ml IFN_γ over 4 days in PC-3 and PC-3 (pRc/RSV-p300) cells was determined by densitometric scanning of radiographic film.

Effect of p300 on IFNγ Antiproliferative Activity

Considering that the overexpression of p300 in PC-3 cells abrogated IFNγ-induced downregulation of neu/HER-2 following IFNγ treatment we wanted to determine if the overexpression of p300 interfered with the antiproliferative effects of IFNγ. Transfected PC-3 cells were treated with or without IFNγ at concentrations of 5000 and 20000 units/ml for a period of 72 hours. Cells were then harvested and antiproliferative activity was determined by direct cell count. Transfected cell growth was much less inhibited following treatment with 5000 units/ml IFNγ as compared to wildtype PC-3 cells with inhibition values of 7.1% versus 50.8%, respectively (Table 3-13). Interestingly, treatment of transfected cells with 20000 units/ml IFNγ inhibited cell growth by 44.6%, which is comparable to maximum inhibition levels seen in wildtype PC-3 cells. This correlates with the increase in phosphorylated STAT1 production following treatment of transfected PC-3 cells with 20000 units/ml IFNγ (Figure 3-24). Thus, the antiproliferative effect of IFNγ is significantly reduced in PC-3 cells overexpressing p300, but is restored at higher concentrations of IFNγ. This suggests a dynamic and reversible interaction between IFNγ-induced phosphorylated STAT1 and p300.

Effect of p300 on IFNγ-Induced $p21^{WAF1/CIP1}$ Production

We have previously shown that the CKI $p21^{WAF1/CIP1}$ appears to play a role in mediating the antiproliferative effects of IFNγ. As IFNγ has reduced antiproliferative activity on PC-3 cells overexpressing p300, we determined the ability of IFNγ to upregulate $p21^{WAF1/CIP1}$ in transfected PC-3 cells. p300 transfected PC-3 cells were treated with or without IFNγ at concentrations of 5000 and 20000 units/ml over a

Table 3-13: Inhibition of PC-3 (pRc/RSV-p300) Cell Growth by IFNγ

IFNγ (U/ml)	Inhibition (%)
0	--
5000	7.1 ± 0.4
20000	44.6 ± 1.9

[a]Cells were plated into 6 well plates (1×10^5 cells/well), grown in the presence or absence of IFNγ for 72 hours and total number of cells determined. The percent viability was approximately 91%. Data are from three representative experiments performed in triplicate. Statistical significance was shown by Student's *t*-test for the difference in cell number grown in the presence and absence of 20000 units/ml IFNγ ($p<0.05$). Wildtype PC-3 cells were inhibited by 50.8% following treatment with 5000 units/ml IFNγ.

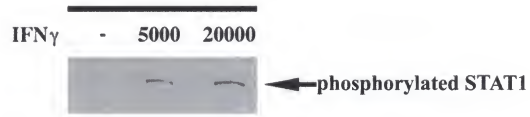


Figure 3-24: IFNγ Induces STAT1 Phosphorylation in PC-3 (pRc/RSV-p300) Cells in a Dose-Dependent Manner. Total cell lysate from PC-3 (pRc/RSV-p300) cells treated with or without 5000 and 20000 units/ml IFNγ for 10 minutes were immunoprecipitated using anti-phosphorylated STAT1 antibodies. The immunoprecipitates were then subjected to immunoblotting using antibodies for phosphorylated STAT1.

time period of 16 and 24 hours. Cells treated for 24 hours with 5000 and 20000 units/ml IFNγ showed a 1.5 and 3.7-fold increase in $p21^{WAF1/CIP1}$ production, respectively, as determined by densitometric analysis (Figure 3-25). No $p21^{WAF1/CIP1}$ was detected at 16 hours. Wildtype PC-3 cells treated with 5000 units/ml IFNγ over 24 hours showed a 2.5-fold increase in $p21^{WAF1/CIP1}$ production. Thus, treatment of transfected PC-3 cells with 5000 units/ml IFNγ led to decreased $p21^{WAF1/CIP1}$ production as compared to wildtype PC-3 cells, correlating with the reduced antiproliferative effects of IFNγ on transfected PC-3 cells. Consistent with the antiproliferative effects of IFNγ on transfected PC-3 cells, increasing IFNγ treatment to 20000 units/ml, thereby increasing phosphorylated STAT1 levels, was able to restore $p21^{WAF1/CIP1}$ levels to those seen following IFNγ treatment of wildtype PC-3 cells. Therefore, IFNγ treatment inhibits the growth of prostate cancer cells correlating with the downregulation of neu/HER-2 expression, which may be mediated by phosphorylated STAT1 binding to the transcriptional co-activator p300.

Superantigen Amplification of Anti-Tumor Immunity

Vaccination Followed By Superantigen Administration Elicits Anti-Tumor Protection

We began our study by determining the ability of superantigens to enhance an ineffective anti-tumor immune response leading to effective anti-tumor protection. Mice were vaccinated with irradiated B16F10 melanoma cells at day 0 and subsequently treated with 25 μg each SEA and SEB at either day 6 or day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged with viable B16F10 cells on day 13. Mice receiving

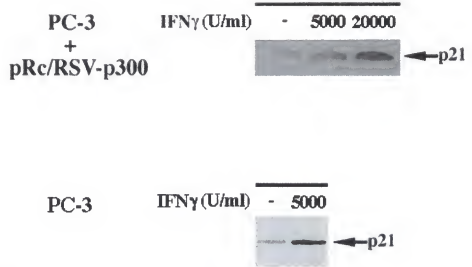


Figure 3-25: IFNγ Upregulates $p21^{WAF1/CIP1}$ Expression in Wildtype PC-3 and PC-3 (pRc/RSV-p300) Cells. Wildtype PC-3 and PC-3 (pRc/RSV-p300) cells were treated with 0, 5000, or 20000 units/ml IFNγ for 16 and 24 hours. Cell lysates were then immunoblotted with antibodies specific for $p21^{WAF1/CIP1}$. **(a)** Densitometric analysis of radiographic film showed a 1.5 and 3.7-fold increase in $p21^{WAF1/CIP1}$ expression following IFNγ treatment of PC-3 (pRc/RSV-p300) cells with 5000 and 20000 units/ml for 24 hours, respectively. No increase in $p21^{WAF1/CIP1}$ expression was seen at 16 hours. **(b)** Wildtype PC-3 cells showed a 1.2 and 2.5-fold increase in $p21^{WAF1/CIP1}$ expression following IFNγ treatment for 16 (not shown) and 24 hours, respectively, as determined by densitometric analysis.

vaccination and SEA/SEB survived significantly longer (+150 days) than mice receiving either no treatment, vaccination only, or SEA/SEB only (36 days) (Figure 3-26). This anti-tumor protection appeared to be dependent on the timing of SEA/SEB administration following vaccination as mice receiving SEA/SEB 6 days following vaccination had a 20% survival rate, whereas mice receiving SEA/SEB 10 days following vaccination showed 60% survival. Further, mice receiving SEA/SEB at less than 6 or greater than 10 days following vaccination did not show as significant increases in survival (data not shown). Thus, vaccination followed by superantigen administration was able to elicit potent anti-tumor protection.

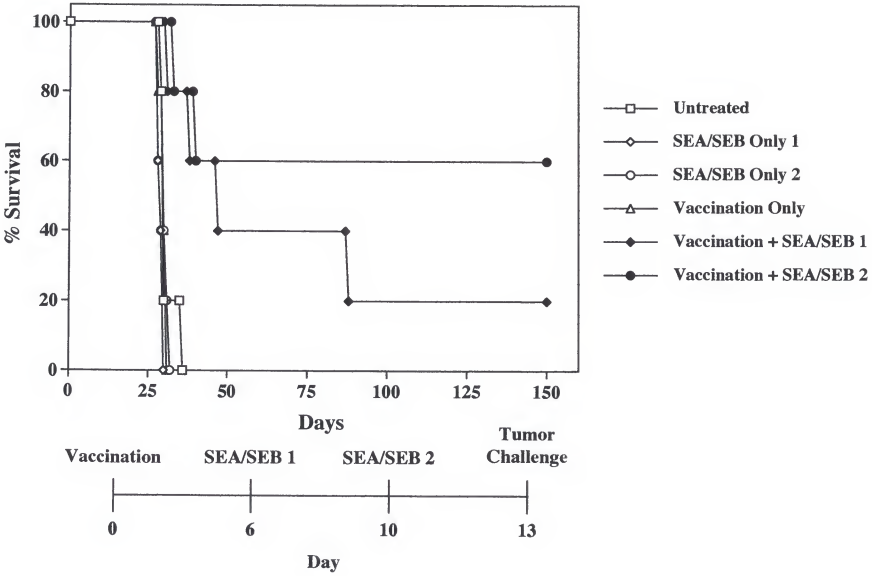
Protection of Long Term Survivors Against Rechallenge With Viable Tumor

Next, we wanted to determine if the protection induced by vaccination combined with superantigen treatment was able to protect surviving mice from a secondary tumor challenge. Mice surviving over 150 days following primary tumor challenge were rechallenged with viable tumor and survival was compared to naïve mice receiving the same tumor challenge dose. Interestingly, the majority of rechallenged mice remained tumor-free, while naïve mice did not survive beyond 14 days (Figure 3-27). This suggests that vaccination followed by superantigen administration not only elicits a potent and protective anti-tumor immune response, but also results in anti-tumor immunological memory.

Effect of Superantigen Dose on Protection Against Tumor Challenge

We next determined the effect of the dose of superantigen administered on eliciting anti-tumor protection. Mice were vaccinated with irradiated B16F10 cells

Figure 3-26: Protection of Mice Against Tumor Challenge is Dependent on the Timing of Superantigen Administration Following Vaccination. C57BL/6 mice (5 per group) were vaccinated i.p. with 1 x 10^6 irradiated B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at either day 6 or 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged i.p. with 2.5 x 10^5 live B16F10 cells at day 13. Mice were evaluated on a daily basis and sacrificed when moribund.


100
80
60
40
20
0
% Survival
0
25
50
75
100
125
150
Days
Untreated
SEA/SEB Only 1
SEA/SEB Only 2
Vaccination Only
Vaccination + SEA/SEB 1
Vaccination + SEA/SEB 2
Vaccination
SEA/SEB 1
SEA/SEB 2
Tumor Challenge
0
6
10
13
Day

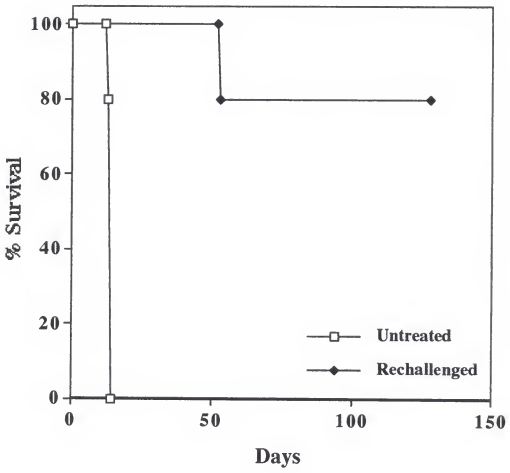


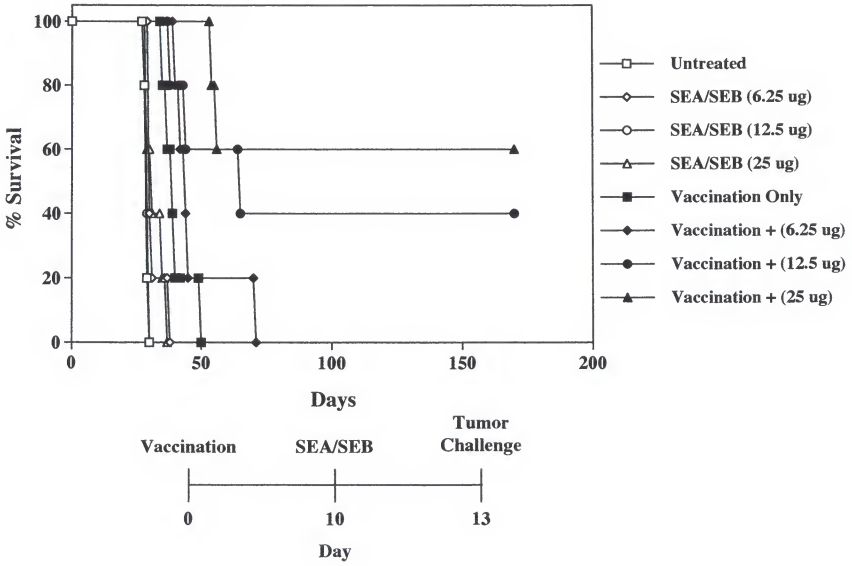
Figure 3-27: Protection of Long Term Survivors Against Rechallenge With Live B16F10 Melanoma Cells. C57BL/6 mice (5 per group) surviving beyond 150 days following vaccination, superantigen administration, and subsequent live B16F10 tumor challenge were rechallenged i.p. with 1 x 10^4 live B16F10 cells at day 0. Mice receiving no treatment served as control. Mice were evaluated on a daily basis and sacrificed when moribund.

at day 0 and subsequently treated with either 6.25, 12.5, or 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged with viable B16F10 on day 13. Consistent with our previous findings, mice receiving vaccination and SEA/SEB survived significantly longer than controls. Furthermore, this prolonged survival was dependent on the dose of SEA/SEB administered (Figure 3-28). Mice receiving 25 and 12.5 μg SEA/SEB showed a 60% and 40% survival rate, respectively. Mice receiving only 6.25 μg SEA/SEB survived significantly longer than controls, but did not have any survivors beyond 71 days. Therefore, vaccination followed by superantigen administration amplified anti-tumor protection in a dose-dependent manner.

Expansion of $CD4^+$, $CD8^+$, and Natural Killer Cells Following Vaccination and Superantigen Administration

T cells and natural killer (NK) cells are classical mediators of cytolytic activity. Thus, we wanted to determine if these cells were responding in the protective anti-tumor effect seen following vaccination and superantigen administration. Mice were vaccinated with irradiated B16F10 cells at day 0 and subsequently treated with 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged with viable B16F10 cells at day 13. Splenocytes were harvested at day 13 and day 27, stained with anti-$CD4^+$, $CD8^+$, or NK antibody, and analyzed by flow cytometry. At day 13, $CD4^+$ levels were similar in all groups, however, mice receiving vaccination and/or SEA/SEB showed increased levels of $CD8^+$ cells as

Figure 3-28: Protection of Mice Against Tumor Challenge is Dependent on Dose of Superantigen Administered Following Vaccination. C57BL/6 mice (5 per group) were vaccinated i.p. with 1 x 10^6 irradiated B16F10 melanoma cells at day 0 and subsequently injected i.p. with either 6.25, 12.5, or 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged i.p. with 2.5 x 10^5 live B16F10 cells at day 13. Mice were evaluated on a daily basis and sacrificed when moribund.


100
80
60
40
20
0
% Survival
0
50
100
150
200
Days
Untreated
SEA/SEB (6.25 ug)
SEA/SEB (12.5 ug)
SEA/SEB (25 ug)
Vaccination Only
Vaccination + (6.25 ug)
Vaccination + (12.5 ug)
Vaccination + (25 ug)
Vaccination
SEA/SEB
Tumor Challenge
0
10
13
Day

compared to mice receiving no treatment or vaccination alone (Table 3-14). Further, mice receiving both vaccination and SEA/SEB also showed an approximately 2-fold increase in NK cells as compared to controls. At day 27, $CD4^+$ and $CD8^+$ levels in mice treated with SEA/SEB alone declined, consistent with the classical deletion seen in T cells activated by superantigen. On the other hand, mice receiving both vaccination and superantigen showed further increased levels of $CD4^+$ and $CD8^+$ cells as compared to controls. This is consistent with the protective anti-tumor response seen in these mice and suggests that superantigen treatment following vaccination does not lead to T cell deletion. Further, mice receiving no treatment, vaccination only, and SEA/SEB only showed a much greater percentage of NK cells than mice receiving both vaccination and SEA/SEB. This suggests that the immune responses of the control groups were largely non-specific, which may explain the morbidity seen in these groups shortly after day 27. Thus, vaccination followed by superantigen administration leads to a significant increase in $CD4^+$ and $CD8^+$ cells as well as an early increase in NK cells, which may all play a role in the resulting potent anti-tumor response.

Vaccination Followed by Superantigen Administration Leads to Increased CTL Activity

We next wanted to determine if vaccination combined with superantigen administration resulted in increased cytotoxic lymphocyte (CTL) activity. Mice were vaccinated with irradiated B16F10 cells at day 0 and treated with 25 μg each SEA and SEB at day 6. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. Splenocytes were harvested at day 9, T cells were

Table 3-14: Expansion of $CD4^+$, $CD8^+$, and NK Cells Following Vaccination and Superantigen Treatment[a]

		%$CD4^+$		%$CD8^+$		%NK	
Group	Day:	13	27	13	27	13	27
Untreated		17.1	10.9	10.3	9.4	2.5	16.0
Vaccination Only		17.8	32.1	10.1	26.1	1.8	13.4
SEA/SEB Only		18.0	14.0	17.3	4.1	2.7	25.4
Vaccination + SEA/SEB		16.5	35.6	15.8	39.5	4.5	6.3

[a]C57BL/6 mice were vaccinated i.p. with 1 x 10^6 irradiated B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged i.p. with 2.5 x 10^5 live B16F10 cells at day 13. Splenocytes were harvested at appropriate time points. An equal number of lymphocytes were stained with anti-$CD4^+$, $CD8^+$, and NK antibodies and subsequently analyzed by flow cytometry.

isolated, and CTL activity was determined by standard 4 hour ^{51}Cr release assay. We found that vaccination followed by SEA/SEB treatment significantly enhanced CTL activity with 85.2% lysis as compared to mice receiving no treatment (0%), vaccination only (2.8%), and SEA/SEB only (27.5%) (Figure 3-29). This is consistent with the increased expansion of T cells in mice receiving both vaccination and superantigen treatment. Thus, vaccination followed by superantigen administration results in increased CTL activity, correlating with the potent anti-tumor response in these mice.

Effect of Superantigen Administration on Serum Levels of IFNγ

IFNγ is well known for its immunoregulatory activities and is mainly produced by T cells and NK cells. It can play an important role in immune responses in activating NK cells and macrophages, enhancing MHC class I and II expression, and shaping T cell responses. Thus, we wanted to determine the effect of superantigen administration on the production of IFNγ. Mice were injected with or without 25 μg each SEA and SEB. Serum samples were then taken over a period of 48 hours and IFNγ levels were determined by ELISA. Mice treated with SEA/SEB showed the highest levels of IFNγ (696 pg/ml) at 6 hours and declined steadily to 0 pg/ml at 48 hours (Figure 3-30). No IFNγ was detected is mice receiving no treatment. Therefore, administration of SEA/SEB leads to a significant increase in IFNγ production, which may play a role in the observed anti-tumor response.

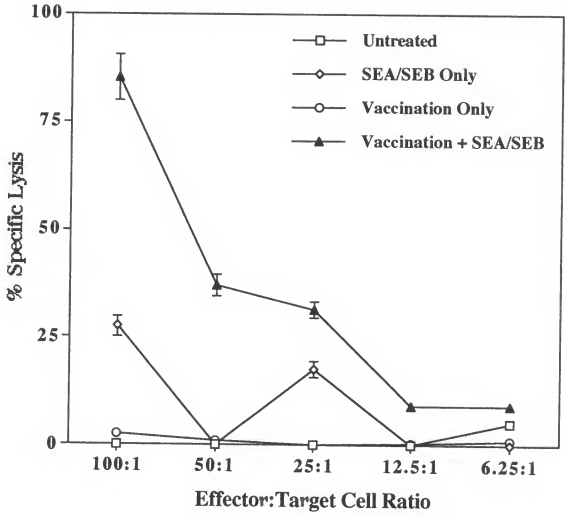


Figure 3-29: Vaccination Followed by Superantigen Administration Results in Increased Tumoricidal Activity. C57BL/6 mice were vaccinated i.p. with 1 x 10^6 irradiated B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at day 6. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. Splenocytes were harvested, T cells were isolated, and CTL activity was determined against ^{51}Cr-labeled B16F10 cells by standard 4 hour ^{51}Cr release assay performed in triplicate.

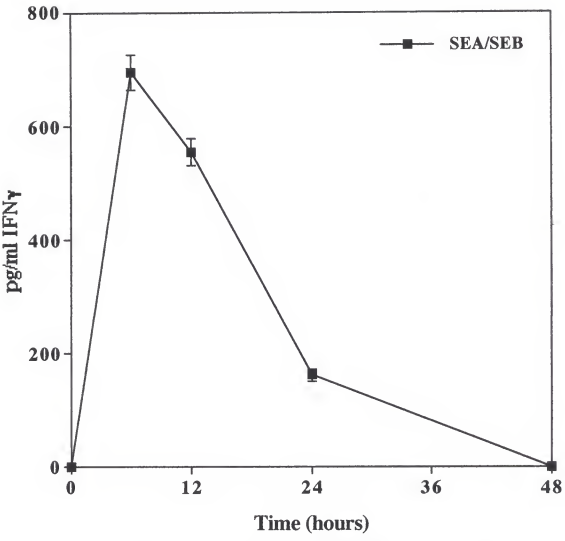


Figure 3-30: Superantigen Administration Results in Increased Serum Levels of IFNγ. C57BL/6 mice were injected i.p. with 25 μg each SEA and SEB. Serum samples were subsequently collected at appropriate time points and IFNγ levels were determined in triplicate by ELISA. No IFNγ was detected in serum from mice receiving no treatment. (43 pg is approximately equal to 1 unit IFNγ.

IFNγ Inhibition of B16F10 Cell Growth

IFNγ is not only known for its immunoregulatory activity, but its anticellular effects are also well documented. Thus, we wanted to determine the antiproliferative effect IFNγ on B16F10 cells in vitro. Cells were treated with or without IFNγ at concentrations ranging from 0.1 to 10000 units/ml over a period of 72 hours. The cells were harvested at appropriate time points and the total number of live cells was determined by direct cell count. B16F10 cells were found to be highly sensitive to IFNγ as cell growth was inhibited by 22.9% with as little as 0.1 units/ml IFNγ (Figure 3-31). Further, treatment with only 10 units/ml IFNγ resulted in near maximal inhibition of cell growth at 71.6%. The ability of SEA/SEB to increase IFNγ levels combined with the sensitivity of B16F10 to the antiproliferative effects of IFNγ suggests that IFNγ may play a role in inhibiting B16F10 cell growth in vivo.

Induction of the Tumor Suppressor Gene Product $p21^{WAF1/CIP1}$

We have shown that IFNγ treatment of various glioblastoma and prostate cancer cell lines led to the upregulation of the tumor suppressor gene product $p21^{WAF1/CIP1}$, describing a mechanism by which IFNγ inhibits cancer cell growth. As B16F10 cells were highly sensitive to the antiproliferative effects of IFNγ we wanted to determine the ability of IFNγ to induce $p21^{WAF1/CIP1}$ in these cells. B16F10 cells were treated with or without 10 units/ml IFNγ over a period of 4-16 hours. Immunoblot analysis using anti-$p21^{WAF1/CIP1}$ antibody on cell lysates showed increased $p21^{WAF1/CIP1}$ production in IFNγ-treated cells as compared to untreated cells at 4, 8, and 12 hours (Figure 3-32). $p21^{WAF1/CIP1}$ levels in IFNγ-treated cells dropped to those seen in untreated cells at 16 hours (data not shown). Thus, IFNγ-

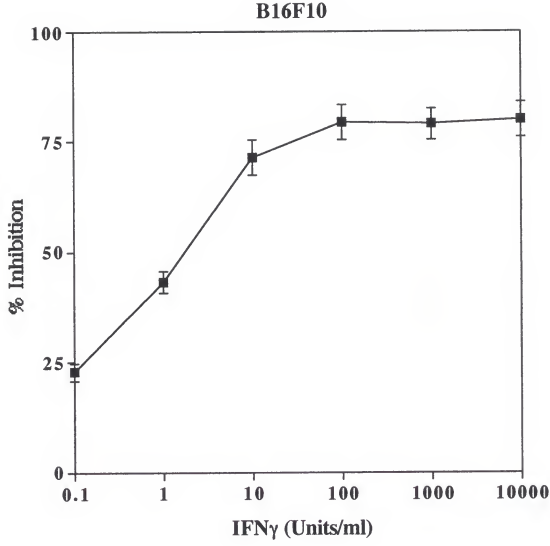


Figure 3-31: IFNγ Inhibits Cellular Proliferation of B16F10 Melanoma Cells in a Dose-Dependent Manner In Vitro. Cells (1 x 10^4 cells/well) were treated with or without IFNγ at concentrations ranging from 0.1 to 10000 units/ml for 72 hours. Cells were then harvested and the total number of live cells was determined by direct cell count. Cell viability was approximately 88%. Data from three representative experiments are expressed as total number of cells per sample +/- S.D. Statistical significance was shown by Student's t-test between the number of cells in the presence and absence of IFNγ ($P<0.04$).

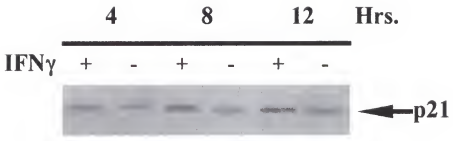


Figure 3-32: IFNγ Treatment Increases $p21^{WAF1/CIP1}$ Production in B16F10 Melanoma Cells. B16F10 cells were treated with or without 10 units/ml IFNγ over a period of 4 to 16 hours. Cell lysates were then immunoblotted with antibodies specific for $p21^{WAF1/CIP1}$. Densitometric analysis of radiographic film showed increases in $p21^{WAF1/CIP1}$ production of 0.9, 1.7, and 1.9-fold at 4, 8, and 12 hours, respectively, following IFNγ treatment. $p21^{WAF1/CIP1}$ levels in IFNγ-treated cells dropped to those seen in untreated cells at 16 hours (data not shown).

induced inhibition of B16F10 cell growth was accompanied by an increase in $p21^{WAF1/CIP1}$ production further suggesting that the direct anticellular effects of IFNγ may play a role in the inhibition of B16F10 cell growth in vivo.

Vaccination Followed by Superantigen Administration Induces a Specific Immune Response

We next wanted to determine if the strong anti-tumor response elicited following vaccination and superantigen administration was specific for B16F10. We vaccinated mice with irradiated B16F10 cells at day 0 and subsequently injected 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were then challenged with viable Lewis lung carcinoma (LL/2) cells at day 13. Mice receiving both vaccination and superantigen treatment did not survive significantly longer than controls (Figure 3-33). This suggests that vaccination followed by superantigen administration elicits a potent and tumor-specific immune response.

Protection of Mice With Established Tumor by Superantigen

Lastly, we wanted to determine the ability of superantigen administration to protect against an established tumor. Mice were injected with viable B16F10 cells at day 0 and subsequently injected with 25 μg each SEA and SEB at day 6. Mice receiving no treatment served as control. Here we found that administration of SEA/SEB six days following tumor challenge significantly prolonged survival as mice receiving SEA/SEB survived 3 times as long as mice receiving no treatment (Figure 3-34). Administration of SEA/SEB later than 6 days following tumor challenge was not as effective in prolonging survival (data not shown). This may be

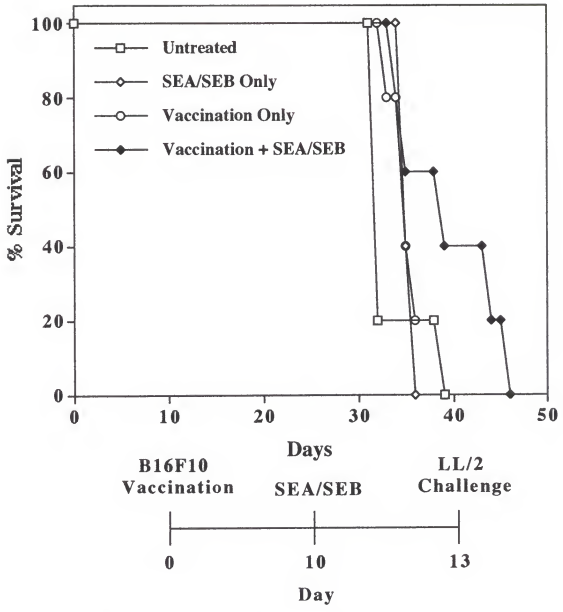


Figure 3-33: Vaccination Followed by Superantigen Administration Induces a Specific Immune Response. C57BL/6 mice (5 per group) were vaccinated i.p. with 1 x 10^6 irradiated B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at day 10. Mice receiving no treatment, vaccination only, and SEA/SEB only served as controls. All mice were challenged i.p. with 1 x 10^6 live Lewis lung carcinoma (LL/2) cells at day 13. Mice were evaluated on a daily basis and sacrificed when moribund.

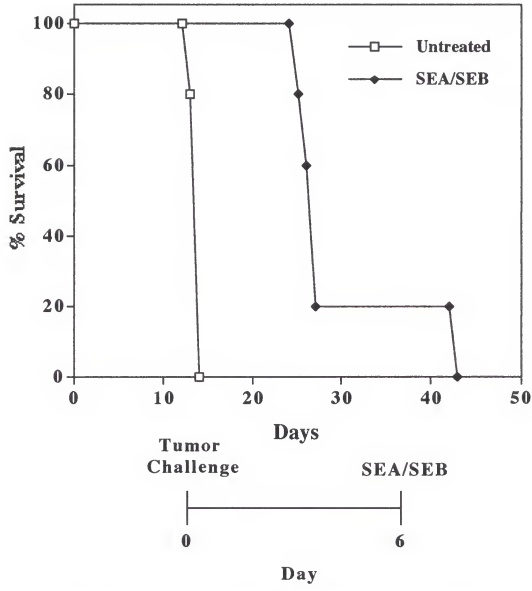


Figure 3-34: Superantigen Administration Prolongs the Survival of Mice With Established Tumor. C57BL/6 mice (5 per group) were challenged i.p. with live B16F10 melanoma cells at day 0 and subsequently injected i.p. with 25 μg each SEA and SEB at day 6. Mice receiving no treatment served as control. Mice were evaluated on a daily basis and sacrificed when moribund.

due to the rapid rate of tumor growth as controls become moribund at 14 days. Thus, SEA/SEB administration prolongs the survival of mice with established tumor.

CHAPTER 4
DISCUSSION

Inhibition of the Glioblastoma Cell Cycle by IFNγ and Acyclovir

IFNγ is a powerful cytokine with a wide range of biological activities. Although interferons are best known for their antiviral activity, they have also been shown to possess potent inhibitory effects on cell growth. IFNγ is also well known for its immunoregulatory activities, including the activation of cytotoxic T cells, natural killer cells, and macrophages, as well as amplification of MHC class I and class II expression. Together, these anticellular and immunoregulatory activities, make IFNγ an attractive anti-tumor agent. Both in vivo and in vitro studies have shown that IFNγ acts directly on tumor cells prolonging their progression through the cell cycle (Fleischman and Fleischman, 1992). Further, indirect immunoregulatory activities of IFNγ have been shown to lead to the targeting of cancer cells by cytolytic immune cells (Baron et al., 1991).

Currently, IFNγ is being used in the treatment of some cancers and is undergoing clinical trials in the treatment of others. However, while IFNγ appears to be an effective treatment for some cancers, it is ineffective on others. As the immunoregulatory mechanisms of IFNγ are fairly well documented, the mechanisms behind the direct anticellular effects of this cytokine are poorly understood. A better understanding of these mechanisms may provide some insight into which cancers

would be responsive to IFNγ treatment allowing the medical community to better serve patients with this disease.

In our studies on the effects of IFNγ on the glioblastoma cell lines T98G, SNB-19, and U-373, we found that IFNγ treatment inhibited the growth of all cell lines leading to a definitive delay in the G_1 to S phases of the cell cycle. Although the cell cycle progression of each cell line is delayed following IFNγ treatment, the cells do continue to slowly progress through the cell cycle. We believe that this may be due to the positive growth signal contributed by the addition of complete media at the time of IFNγ treatment, coupled with the fact that IFNγ effectiveness may diminish over time. Since IFNγ was not repeatedly added throughout the culture period the positive growth signal given by the addition of media may have eventually overcome the negative growth signal given by IFNγ. It is also possible that these cell lines harbor a "leaky" mutation(s), which interferes in the mechanism of IFNγ antiproliferative action.

Investigation into the effects of IFNγ on cell cycle regulators showed that IFNγ treatment increased the production of the CKI $p21^{WAF1/CIP1}$ in all cell lines. Furthermore, the fold increase in $p21^{WAF1/CIP1}$ levels induced by IFNγ relative to controls appeared to coincide with both the degree to which each cell line was inhibited as well as the strength and location of the delay seen in the cell cycle of these cells. Thus, increased levels of $p21^{WAF1/CIP1}$ produced in response to IFNγ appeared to have a direct effect on the cell cycle in all cell lines. These results suggest that $p21^{WAF1/CIP1}$ may play an important role in mediating the effects of IFNγ

on these glioblastoma cell lines, although it is possible that other IFNγ-associated activities contribute to these effects as well.

Although IFNγ treatment led to delays in the cell cycle of all cell lines, the point at which the delays occurred varied. IFNγ treatment of T98G and U-373 cells led to a distinctive G_1 delay, correlating with the kinetics of optimal production of $p21^{WAF1/CIP1}$. On the other hand, SNB-19 cells showed only a slight G_1 delay in response to IFNγ treatment, but did show a substantial S phase block. The absence of a significant G_1 effect, but presence of S phase block appears to correlate with the later kinetics of $p21^{WAF1/CIP1}$ production for this cell line. The CKIs p16 and p27 were not found to play a role in the growth inhibitory effects of IFNγ on the cell lines tested, although other cell cycle players may be involved. $p21^{WAF1/CIP1}$ is also able to inhibit replication by binding to PCNA and preventing it from activating DNA polymerase δ. It has been shown that $p21^{WAF1/CIP1}$ binding to PCNA can lead to delays at both the G_1 and G_2 stages of the cell cycle (Cayrol et al., 1998).

Treatment of glioblastoma multiforme has been largely limited to incomplete surgical resection. Several chemotherapeutic treatments are being used and many more are going through clinical trials, however, none have been highly successful. Thus, the need for new approaches is apparent. Our study shows that IFNγ may be useful in the treatment of glioblastoma as data suggests that IFNγ directly affects the proliferation of glioblastoma cells. Further, due to the direct antiproliferative effect of IFNγ, it may be more effective in treatment if targeted directly to tumor tissue. However, as we have shown that not all glioblastoma cell lines are affected to the same degree, testing IFNγ in tissue culture seems to be a necessary and more rational

approach to the therapeutic treatment of this disease. Thus, in vitro screens of glioblastomas with IFNγ may help determine candidates for targeted administration of IFNγ in therapy.

IFNγ appears to have potential as a treatment for glioblastoma due to its direct antiproliferative effects on these tumors in vitro. However, it has been shown to be toxic in high doses in vivo when given by either the intravenous (i.v.) or intramuscular (i.m.) routes. This toxic effect limits the potential of IFNγ as a treatment for glioblastoma due to the limited dose range that may be used. Administering IFNγ intratumorally could reduce the toxicity of this treatment. This would not only take advantage of the direct anticellular effects of IFNγ, but would also limit the exposure of peripheral tissues to this treatment, thus limiting its toxicity. Another possibility would be to use IFNγ in combination with another anti-tumor agent, which may allow each agent to be used at lower concentrations, thereby reducing toxicity.

In our studies, we determined the effect of IFNγ combined with the nucleoside analog ACV on the glioblastoma cell cycle. Nucleoside analogs are currently being tested in the treatment of several cancers with some success. ACV is being tested in combination with IFN in the treatment of several viral diseases as it is thought to be specifically antiviral due to its need to be phosphorylated by the viral thymidine kinase in order to become active. However, we found that the glioblastoma cell lines T98G, SNB-19, and U-373 were able to phosphorylate ACV. Interestingly, ACV was not phosphorylated as efficiently by normal human astrocytes. In subsequent experiments we found that ACV was able to strongly inhibit the growth of the

glioblastoma cell lines T98G, SNB-19, and U-373 leading to a strong delay beginning at the G_1/S phase transition and continuing throughout the S phase. This correlated with the known ability of ACV to act as a mandatory DNA chain terminator. Moreover, ACV was much less efficient in inhibiting the growth of normal human astrocytes, which is consistent with their lesser ability to phosphorylate ACV.

Our previous studies showed that the CKI $p21^{WAF1/CIP1}$ played a role in mediating the antiproliferative effects of IFNγ on these cell lines through the binding and inhibiting of Cdk2. In further studies into the mechanism of IFNγ action we found that IFNγ-induced $p21^{WAF1/CIP1}$ production resulted in an increase in $p21^{WAF1/CIP1}$-PCNA binding. This correlated with the S phase delay seen following IFNγ treatment. We next tested the effect of IFNγ on normal human astrocytes and found that, similar to ACV, they were much less inhibited by IFNγ than the glioblastoma cell lines.

As both IFNγ and ACV had strong antiproliferative effects on the glioblastoma cell lines tested, which were executed by different mechanisms, they appeared to be good candidates to be used in combination. Our studies did indeed show that the combination of IFNγ and ACV were effective in inhibiting the growth of all glioblastoma cell lines tested in a slightly greater than additive manner. Moreover, by using IFNγ and ACV in combination we were able to reduce the concentrations of each agent without compromising antiproliferative effects. This reduces the potential toxicity of treatment and expands the range of doses that could be used. Further, treatment with either agent alone or in combination did not appear to

significantly affect normal human astrocyte growth. This is significant as intratumoral administration would prevent exposure of peripheral tissues to the treatment, but would not prevent surrounding normal human astrocytes from being exposed. Thus, IFNγ combined with ACV may be an attractive method of glioblastoma treatment, which may be best suited to intratumoral administration due to its direct anti-tumor effects as well as limiting systemic toxicity.

IFNγ Inhibition of Prostate Cancer Cell Proliferation

Type II interferons have been shown to possess antiproliferative effects on various tumor cell types. Further, these antiproliferative effects have been found to correlate with changes in tumor suppressor gene and oncogene expression (Hobeika et al., 1998; Gartel et al., 1996). IFNγ exerts its effects through the JAK/STAT signal transduction pathway in which STAT1 molecules are phosphorylated and translocated to the nucleus where they bind to gamma activated sequences and activate transcription of target genes. Here we have shown that IFNγ exhibits strong antiproliferative effects on the prostate cancer cell lines DU145 and PC-3. Correspondingly, treatment of these cell lines with IFNγ resulted in an increased production of the CKI $p21^{WAF1/CIP1}$ as compared to media control. Interestingly, IFNγ treatment of the prostate cancer cell line LNCaP did not result in any growth inhibition or increase in $p21^{WAF1/CIP1}$ production. Examination of the JAK/STAT signal transduction pathway showed equal amounts of constitutive STAT1 produced in all cell lines following IFNγ treatment. However, IFNγ-treated LNCaP cells failed to produce any phosphorylated STAT1, while significant amounts were produced in both DU145 and PC-3 cells. Independent studies have shown that phosphorylated

STAT1 molecules specifically recognize STAT-responsive elements in the $p21^{WAF1/CIP1}$ promoter and are required for cell growth inhibition in response to IFNγ in various cell lines (Harel-Bellan et al., 1988). This correlates with the lack of antiproliferative activity and failure in $p21^{WAF1/CIP1}$ induction seen in LNCaP cells following IFNγ treatment.

In addition to increasing $p21^{WAF1/CIP1}$ production, we have found that IFNγ treatment of DU145 and PC-3 cells results in a dose-dependent downregulation of the neu/HER-2 proto-oncogene, while this was not seen in LNCaP cells. Recent studies have suggested that the transcriptional co-activator p300 may play a role in the regulation of neu/HER-2. The adenovirus E1A protein was found to transcriptionally repress the neu/HER-2 promoter. Further studies have shown that overexpression of p300 was able to overcome the repression of neu/HER-2 promoter activity by E1A. This repression was also overcome by a p300 mutant deficient in E1A binding. These studies suggest that E1A binding to p300 results in repression of neu/HER-2 promoter activity. In a separate study, phosphorylated STAT1 from IFNγ-treated cell extracts was found to bind to the E1A-binding domain of p300 (Zhang et al., 1996). In light of this evidence we looked at the effect of the overexpression of p300 on IFNγ-induced downregulation of neu/HER-2 expression in PC-3 (pRc/RSV-p300) cells. Interestingly, the overexpression of p300 was sufficient to significantly overcome the repression of neu/HER-2 expression following IFNγ treatment, as was seen in the case of neu/HER-2 repression by E1A. Thus, p300 appears to play a role in the expression of neu/HER-2. Further, IFNγ-mediated repression of neu/HER-2 expression may occur via the interaction of

phosphorylated STAT1 with p300 as phosphorylated STAT1 has been shown to bind to the E1A-binding domain of p300. This provides a mechanism by which IFNγ regulates neu/HER-2 expression and cell growth via phosphorylated STAT1 in addition to $p21^{WAF1/CIP1}$ inhibition of cyclin-dependent kinases.

We subsequently found that the overexpression of p300 greatly reduced the antiproliferative activity of IFNγ on PC-3 (pRc/RSV-p300) cells correlating with the derepression of neu/HER-2. However, this antiproliferative activity was restored following treatment with an increased concentration of IFNγ, thereby increasing the amount of phosphorylated STAT1 available for transcription of target genes. The overexpression of p300 in PC-3 (pRc/RSV-p300) cells also significantly inhibited the ability of IFNγ to increase $p21^{WAF1/CIP1}$ production. Consistent with antiproliferation studies, IFNγ-induced increases in $p21^{WAF1/CIP1}$ production were restored to levels seen in wildtype PC-3 cells following treatment with an increased concentration of IFNγ. Therefore, increased concentrations of IFNγ, and thus increased levels of phosphorylated STAT1, derepressed IFNγ-induced activities requiring phsophorylated STAT1 in cells overexpressing p300. These studies correlate with the proposal of p300-phosphorylated STAT1 binding in response to IFNγ treatment thereby inhibiting the function of both factors. All studies involving PC-3 (pRc/RSV-p300) cells were repeated in PC-3 cells transfected with the empty vector pRc/RSV. The results of these studies showed that PC-3 (pRc/RSV) cells behaved similar to wildtype cells (data not shown).

In summary, we have shown that IFNγ-induced inhibition of prostate cancer cell line growth is accompanied by an increased production of the CKI $p21^{WAF1/CIP1}$

and the decreased expression of the proto-oncogene neu/HER-2. Our findings also implicate the transcriptional co-activator p300 in the positive regulation of neu/HER-2 gene expression. Further, we propose that IFNγ-induced downregulation of neu/HER-2 may involve the binding of phosphorylated STAT1 to p300 thereby inhibiting neu/HER-2 expression.

Superantigen Amplification of Anti-Tumor Immunity

Cancer vaccination or "active immunotherapy" has shown potential as a cancer treatment in both preclinical models as well as early-phase clinical trials. The antigen specificity of the adaptive immune response makes immunotherapy an attractive approach to treating cancer as opposed to the indiscriminate mechanisms of both chemotherapy and radiation therapy. Although cancer vaccines have shown much promise their effectiveness faces some serious obstacles. The identification of several tumor-specific tumor antigens (TSTA) has provided a target against which to vaccinate in a few cancers; however, immunodominant tumor antigens remain to be identified in most cancers. Beyond the identification of TSTAs lies an even greater obstacle: tolerization. Several studies have shown that the efficacy of cancer vaccines is highly limited by the induction of tolerance, which greatly limits the number of tumor-specific T cells activated. This may occur by several mechanisms including, but not limited to insufficient co-stimulatory activity in antigen presentation, delivery of inhibitory signals to T cells via engagement of CTLA-4, or various other mechanisms that limit autoimmune recognition of antigens expressed by normal tissues (Jones et al., 1990; Mueller et al., 1989; Sotomayor et al., 1999).

In the present study, we were able to elicit potent and specific protection against a syngeneic and poorly immunogenic tumor by treating mice with superantigen following vaccination with irradiated tumor cells. In this model, the use of whole tumor cells as a vaccine bypasses the problem of finding TSTAs. However, this does not result in the development of a protective anti-tumor response, although we believe that this does lead to the activation of a small population of tumor-specific T cells, which are then amplified by superantigen to produce effective anti-tumor protection.

Our studies showed that vaccination followed by SEA/SEB administration results in a significant extension of survival as mice receiving both vaccination and SEA/SEB survived over 150 days as compared to only 30 days for controls (mice receiving no treatment, vaccination only, or SEA/SEB only). Further, the majority of mice surviving over 150 days remained tumor free following rechallenge with live tumor cells, suggesting that vaccination followed by superantigen treatment may lead to the development of immunological memory. This anti-tumor protection also appeared to be specific as mice vaccinated with irradiated B16F10 melanoma cells and subsequently treated with SEA/SEB were not protected against a live Lewis Lung carcinoma challenge. The ability of superantigens to enhance a specific immune response has been observed in response to other antigens as well. For example, vaccination with bovine serum albumin (BSA) followed by SEA/SEB administration was found to result in greatly enhanced production of BSA-specific antibody (Torres et al., unpublished data).

As expected, protection against B16F10 tumor cell growth was dependent on the dose of superantigen administered, but was also highly dependent on the timing of superantigen administration in relation to vaccination. Mice treated with SEA/SEB less than 6 days or greater than 10 days following vaccination showed reduced anti-tumor protection. It is possible that the few tumor-specific T cells activated following vaccination may become tolerized over time preventing their further expansion by superantigenic stimulation, as tumor-specific T cell anergy has been shown to be an early event in the tumor-bearing host (Staveley-O'Carroll et al, 1998). It is also possible that superantigenic stimulation of tumor-specific T cells prior to their activation by vaccination could lead to the induction of anergy or deletion, as superantigenic stimulation of naïve T cells is known to lead to an initial V_β-specific expansion of these cells, which is then followed by deletion or anergy. Interestingly, we found that mice receiving both vaccination and SEA/SEB had increased levels of $CD4^+$ and $CD8^+$ T cells detected more than two weeks following SEA/SEB administration as compared to the significant decrease seen in both $CD4^+$ and $CD8^+$ T cell populations in mice receiving SEA/SEB alone. This finding may be explained by two recent studies, which showed that IL-2 could prevent superantigen-induced downregulation of T cell activity (Belfrage et al., 1997; Kuroda et al., 1996). Administration of IL-2 prior to or at the time of SEA administration was shown to prolong the survival of superantigen-reactive T cells. Thus, IL-2 produced by the induction of a minimal immune response following vaccination may provide protection for activated tumor-specific T cells against superantigen-induced downregulation. Further, it has also been shown that CD28 stimulation via B7-1 and

B7-2 accompanied by the production of proinflammatory cytokines such as TNFα and IL-1 may enhance survival of superantigen-stimulated T cells (Vella et al., 1997). These events could be occurring in our tumor vaccine model as well. Lastly, it has been shown that superantigen stimulation of previously activated T cells results in their further expansion, further supporting the large T cell expansion seen following vaccination and SEA/SEB administration (Aoki and Yoshikai, 1997). These findings suggest that superantigen treatment may only be effective in augmenting an anti-tumor immune response during a short window in which tumor-specific T cells are activated and protected from deletion.

As previously mentioned, vaccination followed by SEA/SEB administration led to an increase in both $CD4^+$ and $CD8^+$ T cell populations as compared to controls. In addition, flow cytometry analysis showed an early increase in NK cells in mice receiving both vaccination and SEA/SEB, possibly contributing to anti-tumor protection. However, at later time points the percent of NK cells in control mice far exceeded that seen in mice receiving vaccination followed by SEA/SEB. This suggests that the response of control mice to live tumor challenge was largely non-specific, whereas the response of mice receiving both vaccination and SEA/SEB was dominated by the expansion of T cell subsets.

Consistent with the large expansion of $CD4^+$ and $CD8^+$ T cells seen in mice treated with both vaccination and SEA/SEB, T cells isolated from these mice were efficient in lysing B16F10 melanoma cells in a standard ^{51}Cr release assay as compared to controls. This suggests that cytotoxic T cell activity may play a role in the observed anti-tumor protection. However, this does not rule out the possible role

of $CD4^+$ T cell mediated events such as activation of eosinophils as well as macrophages, which produce both nitric oxide and superoxide. These mechanisms have all been found to play a role in anti-tumor immunity induced by cancer vaccines (Hung et al., 1998).

Another possible player in mediating these anti-tumor effects is IFNγ, whose levels were found to be largely increased following treatment of mice with SEA/SEB. IFNγ not only plays a role in activating cytotoxic T cells, NK cells, and macrophages, but has also been shown to elicit direct anticellular effects on a variety of tumor cell lines (Hobeika et al, 1998; Kominsky et al, 1998). We found that B16F10 melanoma cells were highly sensitive to the direct anticellular effects of IFNγ. Further, IFNγ-induced cell growth inhibition was accompanied by an increased production of the tumor suppressor gene product $p21^{WAF1/CIP1}$. This suggests that the direct anticellular effects of IFNγ may also contribute to the anti-tumor protection seen in response to vaccination followed by superantigen administration.

In addition to being effective in a prophylactic model, superantigen administration was also able to prolong the survival of mice with established tumor in a therapeutic model. At first glance, the anti-tumor protection seen in the therapeutic model does not appear nearly as significant as that seen in the prophylactic model. However, this could be due to the rapid growth of the B16F10 tumor cells, which result in morbidity after only 14 days. The protection against established tumor may be more significant than it appears considering that SEA/SEB

was not administered until day 6 and yet 100% of mice treated with SEA/SEB survived twice as long as controls, with 20% surviving nearly four times as long.

Here we have shown that vaccination with irradiated tumor cells followed by superantigen administration elicited potent and specific protection of mice against challenge with a syngeneic and poorly immunogenic tumor, also leading to the development of immunological memory, which is the ultimate goal of vaccination. Further, superantigen administration alone significantly extended the survival of mice with established tumor. As various cancer patients have been shown to possess small levels of tumor-specific T cells, superantigen may be able to expand this small population to one that is effective in fighting the tumor (Caignard et al, 1994). This study suggests that superantigen has potential in the treatment of cancer via augmenting anti-tumor immunity.

In summary, the studies presented in this dissertation show that both IFNγ and superantigens have potent anti-tumor capabilities. Further, characterization of these anti-tumor effects suggests that they may be mediated through several mechanisms, including the induction of tumor suppressor gene products, downregulation of oncogenes, as well as enhancement of anti-tumor immunity. Taken together, these studies suggest that both IFNγ and superantigens have significant potential in the treatment of various cancers.

REFERENCE LIST

Aaronson, S.A. (1991). Growth factors and cancer. Science 254: 1146-1153.

Adams, P.D., and Kaelin Jr., W.G. (1995). Transcriptional control by E2F. Semin. Can. Biol. 6: 99-108.

Aoki, Y., and Yoshikai, Y. (1997). Further clonal expansion of T cells upon rechallenge of superantigen staphyloccal enterotoxin A. Microbiol. Immunol. 41: 337-343.

Baltimore, D., Berk, A., Darnell, J., Lodish, H., Matsudaira, P., and Zipursky, S.L. (1995). Molecular Cell Biology, 3rd Ed. Scientific American Books Inc. p. 1223.

Baron, S., Tyring, S.K., Fleishmann, W.R., Coppenhaver, D.H., Niesel, D.W., Klimpel, G.R., Stanton, J., and Hughes, T.K. (1991). The interferons: mechanisms of action and clinical applications. J. Amer. Med. Assoc. 266: 1375-1383.

Basham, T.Y., and Merigan, T.C. (1983). Recombinant interferon-γ increases HLA-DR synthesis and expression. J. Immunol. 130: 1492-1494.

Beijersbergen, R., and Bernards, R. (1996). Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochem. Biophys. Acta. 1287: 103-120.

Belfrage, H., Dohlsten, M., Hedlund, G., and Kalland, T. (1997). Prevention of superantigen-induced down-regulation of T-cell mediated cytotoxic activity by IL-2 in vivo. Immunology 90: 183-188.

Bergdoll, M.S. (1985). The staphylococcal enterotoxins-an update. In: Jelaszewics, J., Ed. The Staphylococci. New York: Gustav Fisher Verlag pp. 247-266.

Bishop, J.M. (1991). Molecular themes in oncogenesis. Cell 64: 235-248.

Bussemakers, M.J., van Bokhoven, A., Verhaegh, G.W., Smit, F.P., Karthaus, H.F., Schalken, J.A., Debruyne, F.M., Ru, N., and Isaacs, W.B. (1999). DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res. 59: 5975-5979.

Caignard, A., Dietrich, P.Y., Morand, V., Lim, A., Pannetier, C., Leridant, A.M., Hercend, T., Even, J., Kourilsky, P., and Triebel, F. (1994). Evidence for T-cell clonal expansion in a patient with squamous cell carcinoma of the head and neck. Cancer Res. 54: 1292-1297.

Cavenee, W.K., and White, R.L. (1995). The genetic basis of cancer. Sci. Am. 272: 72-79.

Cayrol, C., Knibiehler, M., and Ducommun, B. (1998). p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene 16: 311-320.

Chen, H., and Hung, M-C. (1997). Involvement of co-activator p300 in the transcriptional regulation of the HER-2/neu gene. J. Biol. Chem. 272: 6101-6104.

Chin, Y.E., Kitagawa, M., Su, W-C., You, Z-H., Iwamoto, Y., and Fu, X-Y. (1996). Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21WAF1/CIP1 mediated by STAT1. Science 272: 719-722.

Cole, B.C., and Griffiths, M.M. (1993). Triggering and exacerbation of autoimmune arthritis by the Mycoplasma arthriditis superantigen MAM. Arth. Rheum. 36: 994-1002.

Cooper, G.M. (1997). The Cell. ASM Press, Washington, D.C.

Darnell Jr., J.E., Kerr, I.M., and Stark, G.R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421.

Dyson, N., and Harlow, E. (1992). Adenovirus E1A targets key regulators of cell proliferation. Cancer Surv. 12: 161-195.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B., and Livingston, D.M. (1994). Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. 8: 869-884.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817.

Farrar, M.A., and Schreiber, R.D. (1993). The molecular cell biology of interferon-γ and its receptor. Annu. Rev. Immunol. 11: 571-611.

Fleischman, W.R., and Fleischman, C.M. (1992). Mechanisms of interferon's antitumor actions. In: S. Baron, D.H. Coppehaver, F. Dianzani, W.R. Fleischman Jr., T.K. Hughes Jr., G.R. Klimpel, D.W. Niesel, G.J. Stanton, S.K. Tyring (eds.) Interferon: Principals and medical applications. Galveston: The University of Texas Medical Branch, pp. 299-309.

Fleisher, B., and Schrezenmeier, H. (1988). T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex Class II molecules on accessory or target cells. J. Exp. Med. 167: 1697-1707.m

Friedman, R.L., Manly, S.P., McMahon, M., Kerr, I.M., and Stark, G.R. (1984). Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell 38: 745-755.

Fuse, T., Yamada, K., Asai, K., Kato, T., and Nakanishi, M. (1996). Heat shock-mediated cell cycle arrest is accompanied by induction of p21 CKI. Biochm. and Biophys. Res. Com. 225: 759-763.

Gajewski, T.F., and Fitch, F.W. (1988). Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. 140: 4245-4252.

Garnick, M.B. (1994). The dilemmas of prostate cancer. Sci. Am. 270: 72-81.

Gartel, A.L., Serfas, M.S., and Tyner, A.L. (1996). p21-negative regulator of the cell cycle. Proc. Soc. Exp. Biol. and Med. 213: 138-149.

Gauci, L. (1987). Management of cancer patients receiving interferon alpha-2a. Int. J. Cancer supp. 1: 21-30.

Giordano, A., and Avantaggiati, M.L. (1999). p300 and CBP: partners for life and death. J. of Cell. Phys. 181: 218-230.

Grana, X., and Reddy, E.P. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (cdks), growth suppressor genes, and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11: 211-219.

Gray, P.W., and Goeddel, D.V. (1982). Structure of the human interferon gene. Nature 298: 859-863.

Gray, P.W., and Goeddel, D.V. (1983). Cloning and expression of murine immune interferon cDNA. Proc. Natl. Acad. Sci. USA 80: 5842-5846.

Greenlund, A.C., Farrar, M.A., Viviano, B.L., and Schreiber, R.D. (1994). Ligand induced IFN-γ receptor tyrosine phosphorylation couples the receptor to its signal transduction system. EMBO J. 13: 1591-1600.

Greenlund, A.C., Morales, M.O., Viviano, B.L., Yan, H., Krolewski, J., and Schreiber, R.D. (1995). Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. Immunity 2: 677-687.

Guy, T.C., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D., and Muller, W.J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA 89: 10578-10582.

Hanada, N., Kido, S., Kuzushima, K., Goto, Y., Rahman, M.M., and Morishima, T. (1989). Combined effects of acyclovir and human interferon-alpha on herpes simplex virus replication in cultured neural cells. J. Med. Virol. 29: 7-12.

Harel-Bellan, A., Brini, A.T., and Farrar, W.L. (1988). IFN-gamma inhibits c-myc gene expression by impairing the splicing process in a colony-stimulating factor dependent murine myeloid cell line. J. Immunol. 141: 1012-1017.

Harrison, P.T., Thompson, R., and Davison, A.J. (1991). Evolution of herpesvirus thymidine kinases from cellular deoxycytidine kinase. Jour. of Gen. Vir. 72: 2583-2586.

Held, W., Acha-Orbea, H., MacDonald, H.R., and Waanders, G.A. (1994). Superantigens and retroviral infection: Insights from mouse mammary tumor virus. Immunol. Today 15: 184-190.

Hirai, H., Roussel, M.F., Kato, J., Ashmun, R., and Sherr, C. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell. Biol. 15: 2672-2681.

Hobeika AC, Etienne W, Cruz PE, Subramaniam PS, Johnson HM. (1998). IFNgamma induction of p21WAF1 in prostate cancer cells: role in cell cycle, alteration of phenotype and invasive potential. Int. J. Cancer 77: 138-145.

Hobeika, A.C., Subramaniam, P.S., and Johnson, H.M. (1997). IFNα induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells. Oncogene 14: 1165-1170.

Howard, A., and Pelc, S.R. (1951). Nuclear incorporation of ^{32}P as demonstrated by autoradiographs. Exp. Cell. Res. 2: 178-187.

Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D., and Levitsky, H. (1998). The central role of $CD4^+$ T cells in the antitumor immune response. J. Exp. Med. 188: 2357-2368.

Hutchinson, C., and Glover, D.M. (1995). Cell Cycle Control. Oxford University Press Inc., New York. pp. 161-163.

Igarashi, K., Garotta, G., Ozmen, L., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Larner, A.C., and Finbloom, D.S. (1994). Interferon-γ induces tyrosine phosphorylation of interferon-γ receptor and regulated association of protein kinases, Jak1 and Jak2, with its receptor. J. Biol. Chem. 269: 14333-14336.

Issacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. Proc. Roy. Soc. London Ser. B. 147: 258-267.

Janeway, C., Lerner, E., Jason, J., and Jones, B. (1980). T lymphocytes responding to mis-locus antigens are lyt-1^+2^- and I-A restricted. Immunogenetics 10: 481-497.

Janknecht, R., and Hunter, T. (1996). Transcription. A growing coactivator network. Nature 383: 22-23.

Jiang, H., Lin, J., Su, Z., Collart, F., Huberman, E., and Fisher, P. (1994). Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 9: 3397-3406.

Jin, X., Nguyen, D., Zhang, W., Kyritsis, A., and Roth, J. (1995). Cell cycle arrest and inhibition of tumor cell proliferation by the p16INK4 gene mediated by an adenovirus vector. Cancer Res. 55: 3250-3253.

Johnson, H.M., Bazer, F.W., Szente, B.S., and Jarpe, M.A. (1994). How interferons fight disease. Sci. Am. 270: 68-75.

Jones, L.A., Chin, L.T., Longo, D.L., and Kruisbeek, A.M. (1990). Peripheral clonal elimination of functional T cells. Science 250: 1726.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311.

Kaufmann, W., and Paules, R. (1996). DNA damage and cell cycle checkpoints. FASEB 10: 238-247.

Kawasaki, H., Carrera, C.J., and Carson, D.A. (1992). Quantitative immunoassay of human deoxycyridine kinase in malignant cells. Anal. Biochm. 207: 193-196.

Kim, T.K., and Maniatis, T. (1996). Regulation of interferon-γ-activated STAT1 by the ubiquitin-proteasome pathway. Science 273: 1717-1719.

Kleihues, P., Soylemezoglu, F., Schauble, B., Scheithauer, B.W., and Burger, P.C. (1995). Histopathology, classification, and grading of gliomas. Glia 15: 211-221.

Kominsky, S., Johnson, H.M., Bryan, G., Tanabe, T., Hobeika, A.C., Subramaniam, P.S., and Torres, B. (1998). IFNγ inhibition of cell growth in glioblastomas correlates with increased levels of the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$. Oncogene 17: 2973-2979.

Kuroda, K., Yagi, J., Imanishi, K., Yan, X.J., Li, X.Y., Fujimaki, W., Kato, H., Miyoshi-Akiyama, T., Kumazawa, Y., Abe, H., and Uchiyama, T. (1996). Implantation of IL-2-containing osmotic pump prolongs the survival of superantigen-reactive T cells expanded in mice injected with bacterial superantigen. J. Immunol. 157: 1422-1431.

Larner, A.C., Chaudhuri, A., and Darnell Jr., J.E. (1986). Transcriptional induction by interferon. New protein(s) determine the extent and length of the induction. J. Biol. Chem. 261: 453-459.

Larner, A.C., Jonak, G., Cheng, Y.S., Korant, B., Knight, E., and Darnell Jr., J.E. (1984). Transcriptional induction of two genes in human cells by beta interferon. Proc. Natl. Acad. Sci. USA 81: 6733-6737.

Levy, D., and Darnell Jr., J.E. (1990). Interferon-dependent transcriptional activation: signal transduction without second messenger involvement? New Biol. 2: 923-928.

Medema, R., Herrera, R., Lam, F., and Weinberg, R. (1995). Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc. Natl. Acad. Sci. USA 92: 6289-6293.

Mollick, J.A., Cook, R.G., and Rich, R.R. (1989). Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. Science 244: 817-820.

Morin, M.D., and Hopkins, W.J. (1998). Treatment of mice with staphylococcal enterotoxin B enhances resolution of an induced Escherichia coli urinary tract infection and stimulates production of proinflammatory cytokines. Infec. and Immun. 66: 2466-2470.

Moul, J.W., Lance, R.S., Theune, S., and Bishoff, J.T. (1992). Probing the molecular machine that drives Pca. Contemp. Urol. 4: 62.

Mueller, D.L., Jenkins, M.K., and Schwartz, R.H. (1989). Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annu. Rev. Immunol. 7: 445.

Nathan, C.F., Murray, H.W., Wiebe, M.E., and Rubin, B.Y. (1983). Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and anti-microbial activity. J. Exp. Med. 158: 670-689.

Nestle, F.O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med. 4: 328-332.

Nishimaki, J., Miyazawa, K., Gotoh, A., Yoshikawa, O., Ohyashiki, K., and Toyama, K. (1996). Inhibitory effect of a nucleoside analog, acyclovir, on leukemia cells. Leuk. Res. 20: 415-420.

Ohgaki, H., Eibl, R.H., Schwab, M., Reichel, M.B., Mariani, L., Gehring, M.,Peterson, I., Holl, T., Weistler, O.D., and Kleihues, P. (1993). Mutations of the p53 tumor suppressor gene in neoplasms of the human nervous system. Mol. Carcinog. 8: 74-80.

Pace, J.L., Russel, S.W., LeBlanc, P.A., and Murasko, D.M. (1985). Comparative effects of various classes of mouse interferons on macrophage activation for tumor cell killing. J. Immunol. 134: 977-981.

Pace, J.L., Russel, S.W., Torres, B.A., Johnson, H.M., and Gray, P.W. (1983). Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. 130: 2011-2013.

Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. (1996). Cancer statistics, 1996. CA Cancer J. Clin. 46: 5-27.

Perussia, B., Kobayashi, M., Rossi, M.E., Anegon, I., and Trinchieri, G. (1987). Immune interferon echances functional properties of human granulocytes: role of Fc receptors and effect of lymphotoxin, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor. J. Immunol. 138: 765-774.

Petroni, K.C., Shen, L., and Guyre, P.M. (1988). Modulation of human polymorphonuclear leukocyte IgG Fc receptors and Fc receptor-mediated functions by IFN gamma and glucocorticoids. J. Immunol. 140: 3467-3472.

Popko, B., Corbin, J.G., Baerwald, K.D., Dupree, J., and Garcia, A.M. (1997). The effects of interferon-gamma on the central nervous system. Mol. Neurobiol. 14: 19-35.

Quesada, J.R., Talpaz, M., Rios, A., Kurzrock, R., and Gutterman, J.U. (1986). Clinical toxicity of interferons in cancer patients: a review. J. Clin. Oncology 4: 234-243.

Reich, N., Evans, B., Levy, D., Fahey, D., Knight Jr., E., and Darnell, J.E. (1987). Interferon-induced transcription of a gene encoding a 15 KDa protein depends on an upstream enhancer element. Proc. Natl. Acad. Sci. USA 84: 6394-6399.

Sala, A., Casella, I., Bellon, T., Calabretta, B., Watson, R., and Peschle, C. (1996). B-myb promotes S phase and is a downstream target of the negative regulator p107 in human cells. J. Biol. Chem. 271: 9363-9367.

Sarup, J.C., and Fridland, A. (1987). Identification of purine deoxyribonucleoside kinases from human leukemia cells: substrate activation by purine and pyrimidine deoxyribonucleosides. Biochemistry 26: 590-597.

Sen, G.C., and Lengyel, P. (1992). The interferon system. J. Biol. Chem. 267: 5017-5020.

Serrano, M., Lee, H., Chin, L., Cordon, C., Beach, D., and DePinho, R. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell 85: 27-37.

Sjoberg, A.H., Wang, L., and Eriksson, S. (1998). Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytostatic and antiviral purine and pyrimidine analogs. Mol. Pharm. 53: 270-273.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and Press, M.F. (1989). Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. Science 244: 707-712.

Slattery, E., Ghosh, N., Samanta, H., and Lengyel, P. (1979). Interferon, double-stranded RNA, and RNA degradation: activation of an endonuclease by (2′-5′)A_n. Proc. Natl. Acad. Sci. USA 76: 4778-4782.

Snapper, C.M., and Paul, W.E. (1987). Interferon gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236: 944-947.

Sotomayor, E.M., Borrello, I., Tubb, E., Allison, J.P., and Levitsky, H.I. (1999). In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails

to prevent the induction of tumor antigen-specific tolerance. Proc. Natl. Acad. Sci. USA 96: 11476-11481.

Spasokoukotskaja, T., Arner, E.S.J., Brosjo, O., Gunven, P., Juliusson, G., Liliemark, J., and Eriksson, S. (1995). Expression of deoxycytidine kinase and phosphorylation of 2-chlorodeoxyadenosine in human normal and tumor cells and tissues. Eur. Jour. of Can. 31A: 202-208.

Spiegel, R.J. (1987). The alpha interferons: clinical overview. Semin. Oncol. 14: 1-12.

Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D., and Levitsky, H. (1998). Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. Proc. Natl. Acad. Sci. USA 95: 1178-1183.

Steinmetz, H.T., Schulz, A., Staib, P., Scheid, C., Glasmacher, A., Neugang, A., Franklin, J., Tesch, H., Diehl, V., and Dias Wickramanayake, P. (1999). Phase-II trial of idarubicin, fludarabine, cytosine arabinoside, and filgrastim (Ida-FLAG) for treatment of refractory, relapsed, and secondaryAML. Ann. Hematol. 78: 418-425.

Stuart-Harris, R.C., Lauchlan, R., and Day, R. (1992). The clinical applications of the interferons: a review. Med. J. Aust. 156: 869-872.

Torrico, F., Heremans, H., Rivera, M.T., Van Marck, E., Billiau, A., and Carlier, Y. (1991). Endogenous IFN-γ is required for resistance to acute Trypanosoma cruzi infection in mice. J. Immunol. 146: 3626-3632.

Trinchieri, G., and Perussia, B. (1985). Immune interferon: a pleiotropic lymphokine with multiple effects. Immunol. Today 6: 131.

Valente, G., Ozmen, L., Novelli, F., Guena, M., Palestro, G., Forni, G., and Garotta, G. (1992). Distribution of interferon-γ receptor in human tissues. Eur. J. Immunol. 22: 2403-2412.

Vella, A.T., Mitchell, T., Groth, B., Linsley, P.S., Green, J.M., Thompson, C.B., Kappler, J.W., and Marrack, P. (1997). CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo. J. Immunol. 158: 4714-4720.

Vial, T., and Descotes, J. (1994). Clinical toxicity of the interferons. Drug Safety 10: 115-150.

Waga, S., Hannon, G., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369: 574-578.

Wheelock, E.F. (1965). Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science 149: 310-311.

Xiong, Y., Hannon, G., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature 366: 701-704.

Yu, D., Suen, T.C., Yan, D.H., Chang, L.S., and Hung, M.C. (1990). Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products. Proc. Natl. Acad. Sci. USA 87: 4499-4503.

Zhang, J.J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C.M., and Darnell Jr., J.E. (1996). Two contact regions between Stat1 and CBP/p300 in interferon γ signaling. Proc. Natl. Acad. Sci. USA 93: 15092-15096.

Zilberstein, A., Kimchi, A., Schmidt, A., and Revel, M. (1978). Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. Proc. Natl. Acad. Sci. USA 75: 4734-4738.

BIOGRAPHICAL SKETCH

Scott Lee Kominsky was born on July 26, 1973, in Highland Park, Illinois, to Allen and Gayle Kominsky. Scott's father Allen was a native of the Chicago area, but his family also spent much time in Miami. Scott's mother Gayle was a native of Wisconsin. In 1978, the Kominsky family moved from the cold weather of Highland Park, Illinois to Miami, Florida. There, Scott attended kindergarten through grade four at Devon Aire Elementary. In 1982 Scott received a blessing in the form of a little sister named Jamee. The following year, Scott and his family moved to Ormond Beach where he attended Ormond Beach Junior High and Seabreeze High School. Scott did very well academically and was admitted to the University of Florida in 1991. Knowing that he wanted to have a career somewhere in the field of health/science he majored in microbiology and in 1995 received his Bachelor of Science degree with a minor in chemistry. During his college career Scott become very interested in the field of immunology. Following his graduation he decided to further his education and was accepted into graduate school in the Department of Microbiology and Cell Science. Here he was kindly taken into the laboratory of Dr. Howard M. Johnson to pursue a doctorate in immunology. Following graduation, Scott will begin a postdoctoral fellowship and he hopes to continue forging a path toward a successful career.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Howard M. Johnson, Chairman
Graduate Research Professor of
Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward M. Hoffman
Professor of Microbiology and Cell
Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Francis C. Davis
Associate Professor of Microbiology
and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

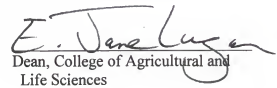
Janet K. Yamamoto
Associate Professor of Immunology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ammon B. Peck
Professor of Pathology,
Immunology, and Laboratory
Medicine

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2000



Dean, College of Agricultural and Life Sciences

Dean, Graduate School